Studies on the selected vernalization pathway, *MADS*-box and *FT* genes to explore their roles in bamboo flowering

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Abstract

Bamboos are the fastest growing grasses of the family Poaceae. Majority of the bamboos have an extreme long vegetative phase compared to their phylogenetically close relatives. In extreme cases, the vegetative phase may be extended upto 150 years before the onset of flowering. Various exogenous factors such as daylength, temperature and endogenous factors such as physiological age, hormones together play important regulatory roles in flowering. Vernalization and ambient temperature pathways are two well-known flowering pathways that demonstrates impact temperature on flowering. During flowering, the signals from all the pathways are integrated by *FT*, which in turn activates *AP1* that belongs to the *MADS*-box gene family to induce flowering. However, in some Poaceae members, *AP1* functions upstream of *FT*. Therefore, the main aim of this study is to identify and expression characterize important genes such as *BtVIL1, BtVIL2, BtVIL3*, *BtVIL4* and *BtGhd7* that are known to be vernalization responsive in Arabidopsis. The study also undertook sequence, domain, syntenic and phylogenetic analyses of the entire *MADS-box* gene family across 11 sequenced bamboo genomes. Finally, this study performed heterologous expression of *BtMADS14*, *BtFT1* and *BtFT3* genes in Arabidopsis to confirm their flower inductive function.

1. Introduction

Bamboos belong to the monocotyledonous plant subfamily Bambusoideae and family Poaceae. Total 1670 bamboo species belonging to 125 genera have been discovered till date (Das et al., 2008; Kelchner, 2013; Soreng et al., 2017). They are the fastest growing plant in the world (Chen et al., 2022). Bamboos can grow a large range of altitude starting from sea level upto 4000 m altitude (Behari, 2006). Bamboo flowering is quite distinct from other monocotyledons (Kurz, 1876; Janzen, 1976), particularly with respect to the extremely long vegetative phase (upto ~150 years) before the onset of flowering. Three distinct types of flowering have been reported in bamboo, which are annual, sporadic and gregarious (Brandis, 1906; Guangchu, 2002).

During flowering, the vegetative shoot apical meristem (SAM) is converted into inflorescence meristem (IM), which eventually turns into floral meristem (FM). Various exogenous and endogenous pathways together control flowering. Among them, photoperiod, vernalization, ambient temperature pathways come under exogenous pathways, while autonomous, gibberellic acid, age pathways fall under endogenous pathways. The floral pathway integrator genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) integrate the signals from various pathways and ultimately initiates flowering.

Exposure to 1-7°C cold temperature for a longer period of time activates the vernalization pathway genes, which induce flowering in the spring season to ensure highest reproductive success (Bernier et al., 1981; Sung and Amasino, 2005; Dennis and Peacock, 2007). However, not much studies were conducted to know the vernalization pathway gene regulations in bamboos till date.

Genetic regulatory networks related to the vernalization pathway have been well studied in the model temperate plant Arabidopsis. Here, central regulatory roles are performed by *VERNALIZATION INSENSITIVE 3* (*VIN3*) and *VERNALIZATION INSENSITIVE 3 LIKE* (*VIL*) genes (*VIL1*, *VIL2*, *VIL3* and *VIL4*). They all contain three domains, which are the plant homeodomain finger (PHD), Fibronectin type 3 domain (FN3) and the VIN3 interacting domain (VID, Fu et al., 2007). Only *VIL4* is exceptional by having only VID domain and thus considered as a pseudogene (Sung et al., 2006). During winter, *VIN3/VIL* is induced, which suppresses *FLOWERING LOCUS C* (*FLC*) by epigenetic modifications. Suppression of *FLC* leads to the activation of downstream floral inducer genes and ultimately flowering occurs in the spring (**Figure 1**, Kim and Sung, 2013).

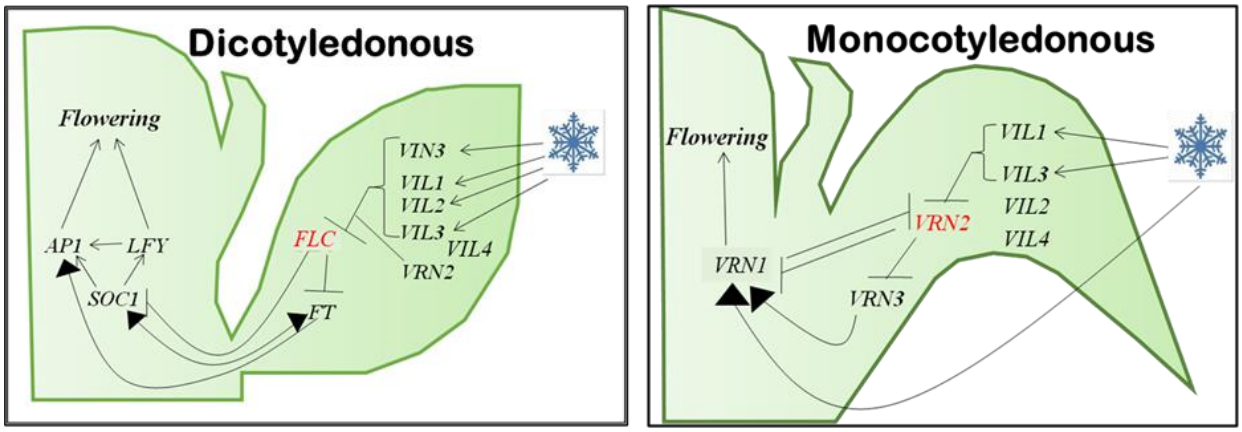

Figure 1. Schematic representation of vernalization responsive genes' regulatory loop identified in Dicotyledonous (*Arabidopsis*) and Monocotyledonous (Wheat) plants. The arrows indicate induction and bars represent suppression.

In monocotyledonous plants, multiple *VIL*s such as in wheat (*TmVIL1-TmVIL3*) and in rice (*OsVIL1-OsVIL4*, Fu et al., 2007). In temperate plants, such as *Triticum monococcum*, *VIL* genes are induced by cold temperature (Sung et al., 2007), while in tropical rice, they are induced by short day (Wang et al., 2013). In monocotyledonous, temperate plants, *VERNALIZATION 2* (*VRN2*) is the functional homolog of *FLC*. Phylogenetic analysis of monocotyledonous *VRN2* like genes suggested occurrence of a major duplication event before diversification of grasses giving rise to *VRN2*/ *Grain number, plant height and heading date 7* (*Ghd7*) and *CONSTANS 9* (*CO9*) clades (Woods et al., 2016). Among them, *VRN2* represses flowering in the temperate plants and such vernalization induced repression of *VRN2* can be seen in core Pooid members, such as wheat and barley (Woods et al., 2016). On the other hand, *Ghd7* is exclusively limited to tropical rice, which is induced by *VIL1* gene in long day or summer to suppress flowering, suggesting its participation in photoperiodic pathway (Jeong et al., 2016; Zheng et al., 2019). During short day/winter, in tropical rice, *VIL* suppresses *LFL1*, and thereby withdraws the suppression on *Ehd1* (Yang et al., 2013). All signals coming from the Vernalization pathway gene ultimately induce *VRN3* (*~FT*) and *VRN1* (*~APETALA1/AP1*) to initiate flowering (**Figure 1**).

In dicotyledonous plants such as Arabidopsis, *FT* functions in the upstream of *AP1* to promote flowering (Abe et al., 2005). In contrast, in core Pooideae member such as wheat, *AP1* functions in the upstream of *FT*. The transcript accumulation of *AP1* leads to accumulation of *FT* and consequently induces flowering (Shimada et al., 2009). *AP1* belongs to the type II *MIKC^C MADS*-

box genes involved in development of sepals in *Arabidopsis* (Mandel et al., 1992). In contrast, overexpression of cucumber and poplar *AP1*-like gene resulted in early flowering in transgenic *Arabidopsis* plants (Zheng et al., 2014; Zhou et al., 2019) suggesting its role in floral induction also. *MADS-box* family transcription factors play essential roles in regulating growth and development of diverse eukaryotic organisms (Rounsley et al., 1995). The acronym MADS was derived from the proteins MINICHROMOSOME MAINTENANCE1 (**M**CM1, Passmore et al., 1988) from *Saccharomyces cerevisiae*, AGAMOUS (**A**G, Yanofsky et al., 1990) from *Arabidopsis thaliana*, DEFICIENS (**D**EFA, Sommer et al., 1990) from *Antirrhinum majus* and SERUM RESPONSE FACTOR (**S**RF, Norman et al., 1988) from *Homo sapiens*.

The *MADS-box* gene family is subdivided into three major groups- *ARG80-like* or *SRF-like* genes, *MEF2-like* genes and *MIKC*-type genes (Theißen and Saedler, 1995; Theißen et al., 1996; Hasebe and Banks, 1997; Münster et al., 1997). *SRF-like* genes, also known as type I genes, are found in fungi, plants and animals. *MEF2-like* genes are animal and fungi specific while *MIKC*-type genes, also known as type II genes, can be found in plants only (Theißen et al., 2000, **Figure 2**). *MIKC*-type genes contain MEF2-like MADS domain (M), intervening domain (I), keratin-like domain (K), and a diverged C-terminal domain (C, De Bodt et al., 2003a, 2003b; Kaufmann et al., 2005; Gramzow and Theißen, 2010). Based on diversity in I and K domains, type II *MADS*-box has been divided into $MIKC^{*}$ and $MIKC^{C}$ types (Henschel et al., 2002). Among various types, type II $MIKC^{C}$ are involved in flower induction and floral organ differentiation (Ren et al., 2021).

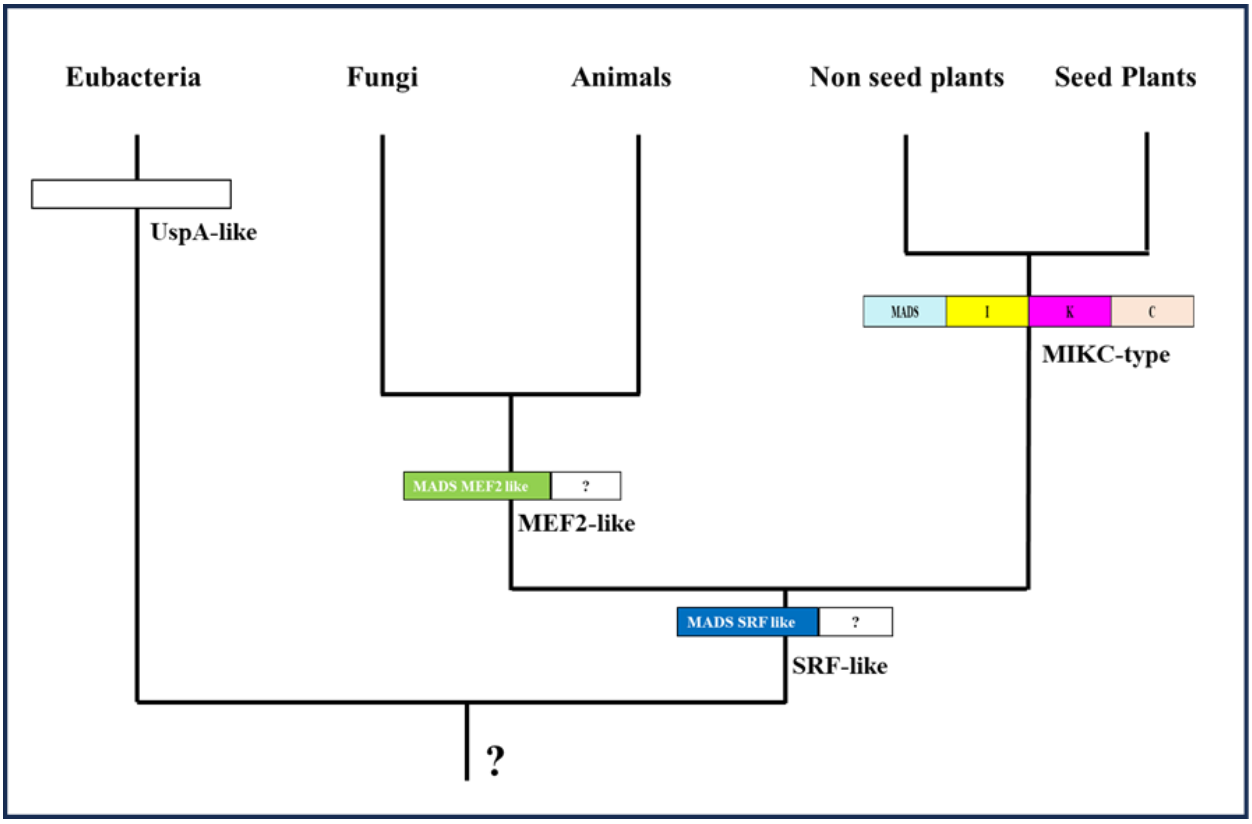

Figure 2. The major clades of *MADS-box* genes in the evolution of life. The figure was redrawn based on the information obtained from Theißen et al., 2000 and Gramzow and Theißen, 2010.

FT is the floral integrator gene, which is synthesized in the leaf and transferred to SAM to induce flowering (Turck et al., 2008; Song et al., 2013). *FT*, *FD* and *14-3-3* together forms a florigen activation complex (FAC) which eventually induces *AP1* and *SOC1* before flowering (Abe et al., 2005; Collani et al., 2019). Ectopic expression of *Arabidopsis FT* on *Manihot esculenta* and *Eucalyptus grandis×urophylla* resulted in early flowering (Klocko et al., 2016; Adeyemo et al., 2017) suggesting its role in flower induction.

Due to lack of sequenced bamboo genomes, very limited studies could have been conducted to study vernalization pathway, *MADS-box* and *FT* genes and their specific functional role in flowering. Presence of 42 (Zhang et al., 2018) *MADS-box* genes in *Phyllostachys edulis* and 170 $MIKC^C$ genes in *Dendrocalamus latiflorus* (Yang et al., 2022) have previously been reported. In the tropical bamboo *Bambusa tulda*, 4 copies of *FT* genes (*BtFT1*, *BtFT2*, *BtFT3* and *BtFT4*) have been identified by Sanger's sequencing and their expression analysis shows involvement of only

BtFT3 and *BtFT4* in flowering (Dutta et al., 2018). Again, deduced amino acid sequence analysis suggested the changes in two amino acids between BtFT1/2 (Phe101>Ile101) and BtFT3/4 (Phe64>Leu64) groups. Whether these two amino acid changes will lead to any functional alteration between the two group of FT genes with respect to flowering is yet to be discovered. Recent availability of the genome and transcriptome of 11 additional bamboo species (http://www.genobank.org/bamboo, Liu et al., 2024; Ma et al., 2024) provide opportunity to initiate comparative evolutionary studies.

In the current study, sequence variations of selected vernalization pathway genes, *MADS-box* and *FT* gene homologs were studied in all sequenced bamboos that underwent different levels of polyplodization, diverse flowering time and growth (**Figure 3**). The bamboos selected for this study were *Olyra latifolia* (Olyrinae, 2n=2x=22, Guo et al., 2019), *Raddia guianensis* (Olyrinae, 2n=2x=22, Guo et al., 2019), *Ampelocalamus luodianensis* (Ampelocalaminae, 2n=4x=48, Ma et al., 2024), *Hsuehochloa calcarea* (Hsuehochloinae, 2n=4x=48, Ma et al., 2024), *Phyllostachys edulis* (Arundinariinae, 2n=4x=48, Zhang et al., 2011), *Guadua angustifolia* (Guaduinae, 2n=4x=46, Guo et al., 2019), *Otatea glauca* (Guaduinae, 2n=4x=48, Ma et al., 2024), *Rhipidocladum racemiflorum* (Arthrostylidiinae, 2n=4x=48, Ma et al., 2024), *Bonia amplexicaulis* (Bambusinae, 2n=6x=72, Guo et al., 2019), *Melocanna baccifera* (Melocanninae, 2n=6x=70, Ma et al., 2024) and *Dendrocalamus sinicus* (Bambusinae, 2n=6x=70, Ma et al., 2024). In addition to these bamboos, *VIL*, *AP1* and *FT* genes have been obtained from *Bambusa tulda* by Sanger's sequencing (Bambusinae, 2n=6x=70/72, aneuploid, Kumar et al., 2011, **Figure 3**).

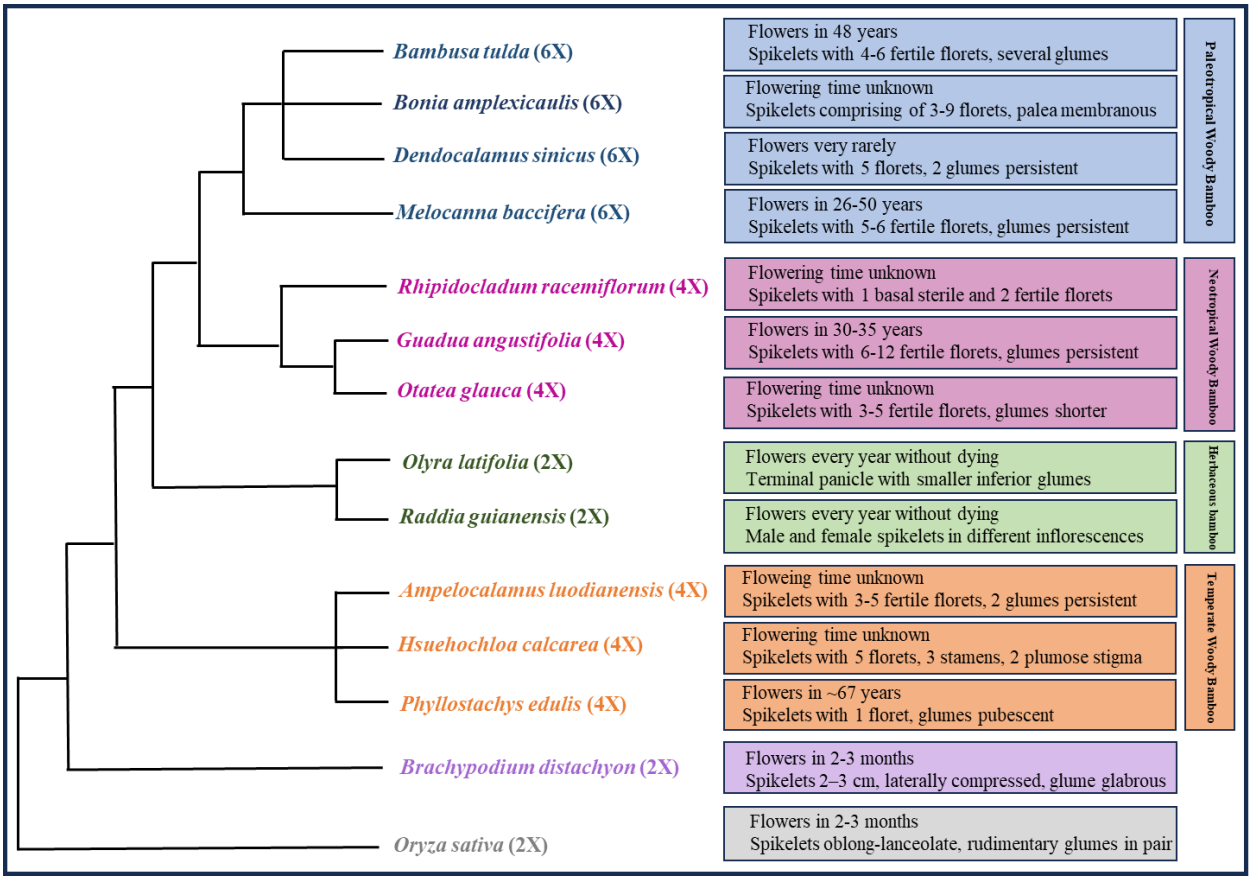
Bambusa tulda (6X)
Bonia amplexicaulis (6X)
Dendocalamus sinicus (6X)
Melocanna baccifera (6X)
Rhipidocladum racemiflorum (4X)
Guadua angustifolia (4X)
Otatea glauca (4X)
Olyra latifolia (2X)
Raddia guianensis (2X)
Ampelocalamus luodianensis (4X)
Hsuehochloa calcarea (4X)
Phyllostachys edulis (4X)
Brachypodium distachyon (2X)
Oryza sativa (2X)
Flowers in 48 years
Spikelets with 4-6 fertile florets, several glumes
Flowering time unknown
Spikelets comprising of 3-9 florets, palea membranous
Flowers very rarely
Spikelets with 5 florets, 2 glumes persistent
Flowers in 26-50 years
Spikelets with 5-6 fertile florets, glumes persistent
Paleotropical Woody Bamboo
Flowering time unknown
Spikelets with 1 basal sterile and 2 fertile florets
Flowers in 30-35 years
Spikelets with 6-12 fertile florets, glumes persistent
Flowering time unknown
Spikelets with 3-5 fertile florets, glumes shorter
Neotropical Woody Bamboo
Flowers every year without dying
Terminal panicle with smaller inferior glumes
Flowers every year without dying
Male and female spikelets in different inflorescences
Herbaceous bamboo
Floweing time unknown
Spikelets with 3-5 fertile florets, 2 glumes persistent
Flowering time unknown
Spikelets with 5 florets, 3 stamens, 2 plumose stigma
Flowers in ~67 years
Spikelets with 1 floret, glumes pubescent
Temperate Woody Bamboo
Flowers in 2-3 months
Spikelets 2–3 cm, laterally compressed, glume glabrous
Flowers in 2-3 months
Spikelets oblong-lanceolate, rudimentary glumes in pair

Figure 3. Phylogenetic relationship of the studied bamboos with monocotyledonous model plants *Brachypodium* and Rice. The ploidy levels of the plants are mentioned in parentheses. The floral morphologies are mentioned for each plant in the right-hand side. The figure was redrawn based on the informations obtained from Soreng et al., 2017; Basak et al., 2021 and Huang et al., 2022.

As mentioned above, till date, 4 *AP1* (*BtMADS14*, *BtMADS15*, *BtMADS18*, *BtMADS20*, Basak et al., 2025) and 4 *FT* homologs (*BtFT1*, *BtFT2*, *BtFT3*, *BtFT4*, Dutta et al., 2018) from tropical bamboo *B. tulda* have been sequenced. Their tissue specific expression analysis by RT-qPCR suggested possible involvement of *BtMADS14*, *BtMADS15*, *BtFT3* and *BtFT4* in flower induction (Dutta et al., 2018; Basak et al., 2025). However, the functional characterizations of these genes have not yet been performed. Also, no studies have so far been conducted to characterize vernalization pathway genes in bamboo. Therefore, the aim of this study is to understand the regulation of the vernalization pathway genes, detailed evolutionary comparisons on *MADS-box* genes across sequenced bamboo species and to undertake functional characterization of *BtFT1*,

BtFT3 and *BtMADS14 in planta* by heterologous expression in Arabidopsis *thaliana*.

2. Objectives (as specified in approved 1000 words)

Objective 1. Identification, sequence characterization and gene expression analyses of important vernalization pathway genes in bamboo

- PCR amplification and sequencing of important vernalization pathway genehomologs in *B. tulda* Roxb. i.e., *BtVIL1*, *BtVIL2*, *BtVIL3*, *BtVIL4* and *BtCO9*
- Bioinformatics comparisons of these gene sequences with homologs identified in the temperate bamboo *Phyllostachys edulis* and other phylogenetically related monocot species
- Phylogenetic analyses of *B. tulda* vernalization genes in comparison to closely related monocot species
- Study on tissue specific expressions of these genes by real time RT-qPCR analyses
- Study on seasonal expression pattern of these genes at regular intervals throughout the year to understand their responsiveness to cold temperature

Objective 2. Genome wide identification, synteny, phylogeny and *in silico* expression analyses of *MADS-box* genes from sequenced bamboo genomes

- Identification of all *MADS-box* containing genes from the sequenced bamboo genomes- *Bonia amplexicaulis*, *Guadua angustifolia*, *Raddia guianensis*, *Olyra latifolia*, *Phyllostachys edulis*
- Phylogenetic relationship and gene copy expansion/reduction analyses compared to closely related monocots
- Domain analysis of putative MADS-box proteins from bamboos
- Synteny, *in silico* expression and gene regulatory network analyses of *P. edulis MADS-box* genes

Objective 3. Ectopic expression analyses of *BtFT1*, *BtFT3* and *BtMADS14* in *Arabidopsis thaliana*

- PCR amplification of *BtMADS14*, *BtFT1* and *BtFT3* genes for Gateway cloning of

BtMADS14, *BtFT1* and *BtFT3* in pDONR221 (BP) and pALLIGATOR2 (LR) vectors

- *In planta* transformation of *BtMADS14*, *BtFT1* and *BtFT3* in *Arabidopsis thaliana* via floral dip method
- Phenotyping of overexpressed transgenic plant lines in comparison to wild type plants
- Expression analysis of downstream target genes in transgenic plants to identify responsive regulatory pathways

3. Methodology

3.1. Retrieving genomic and protein sequences of homologs from sequenced bamboos

Rice protein sequences were used as query to retrieve gene and CDS sequences of homologous genes from all sequenced bamboos from GenDBE (https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi) and Bamboobase (https://bamboo.genobank.org/). The homologous *Brachypodium* genes were retrieved from Plaza_monocots_v5 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/). The BLASTP analysis was carried-out by using the e-value threshold $\leq$ e-65 and identity $\geq$ 50%.

3.2. Designing of primers for gene sequencing and expression analysis

Multiple alignment of the homologous CDS sequences obtained from related monocotyledonous were done using MUSCLE program (https://www.ebi.ac.uk/jdispatcher/msa/muscle). For the PCR amplification, forward and reverse primers were designed from the conserved regions. For gene expression studies, gene specific primers were designed from isolated *B. tulda* exon sequences (**Table 1**). All primers were designed in Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). For gateway cloning, gateway specific tags were added to the primer sequences. For downstream target gene expression analysis in wild type and transgenic Arabidopsis plants, gene specific primers were designed from *A. thaliana* CDS sequences.

Table 1. Primer sequences used for gene amplification and expression analysis. Abbreviations used: bp- base pair, F- forward, R- reverse.

Purpose	Gene name	Primer sequence (5'-3')	Primer length (bp)	GC content (%)	Tm (°C)
	BtVIL1	F- ATGGCGTCGAGCGGAGG	17	70.6	60.7

Gene amplification		R- TTAATGCCATAGCTTAGTGCAGAAA	25	36.0	52.4
Gene expression		F- TGGAAACTGAGGTTGGCCCC	20	60.0	62.4
		R- CAGAGTTTGTAGCCTTTGATAGCA	24	41.7	59.1
Gene amplification	*BtVIL2*	F- ATGGCTACTCGCATTGCBHTC	21	55.0	59.8
		R- TTAATGCCAAAGTTCCATGCA	21	38.1	53.2
Gene expression		F- TCTGGTGGATCTCCTGCTAACT	22	50.0	60.3
		R- AAAACCAGTTACCTCTTGGGCAA	23	43.5	60.6
Gene expression	*BtVIL3*	F- AGTTCTCCAGGCCTGCATC	19	57.9	59.4
		R-CTTTGATGGTTTCAGATGATGCTA	24	37.5	57.3
Gene amplification	*BtVIL4*	F- ATGGATCCAGAAAAACG	17	41.2	47.9
		R- CTAATGCCAAAGCTTGYAGCA	21	47.6	55.0
Gene expression		F- CATTGTGAACCGGCTTAGTTG	21	47.6	57.8
		R- AAGGATTTGGGGTTGGGGA	19	52.6	58.4
Gene amplification	*BtGhd7*	F- ATGTCGGCGGCGTCGG	16	75.0	61.9
		R- CTACGAACGGAACCATCC	18	55.6	52.6
Gene expression		F- GTCTCGCTTGGCAGGGGC	18	72.2	62.0
		R- GTGAATGTGCTCCCACAGAATGACA	25	48.0	59.3
	BtLFL1	F- TCAGAACCCATGATCTTCAACT	22	40.9	57.1
		R- CTTGATCATCTCCCGCCTT	19	52.6	56.9
	BtEhd1	F- AGCTGCAGGGTGGGTTTAA	19	52.6	59.1
		R- TCATATGCAGGCACCAAGTG	20	50.0	58.2
Gene amplification	*BtMADS14*	F-ATGGGGCGCGGGAAGGTG	18	72.2	74.8
		R-CTAGCCGTCGATGTGGCTCACCA	23	50.0	68.9
Gene expression		F-CTCGTGGAGAAGCAGAAGGTC	21	57.1	57.4
		R-CTGCTGCGTCCTCTGCCCT	19	68.4	62.7
Gateway cloning		F-ggggacaagtttgtacaaaaaagcaggcttaATGGGGCGCGGGAAGG	47	53.2	70.5
		R-ggggaccactttgtacaagaaagctgggtTCATCCGTTAATGTGGCTCACCA	52	50.0	70.1
	BtFT1	F-ggggacaagtttgtacaaaaaagcaggcttaATGGTCGGCGGGGACAGG	49	53.1	72.9
		R-ggggaccactttgtacaagaaagctgggTCACCAGGGGTACATCCTTCTT	50	52.0	72.8
	BtFT3	F-ggggacaagtttgtacaaaaaagcaggcttgATGGTCGGCGGGGACAG	48	54.2	73.1
		R-ggggacaactttgtatagaaaagttgTCACCAGGGGTACATCCTTCTTC	49	44.9	69.6
	BtMADS15	F-ATGGGGCGCGGGAAGGTG	18	72.2	74.8

Gene amplification		R- TTAAGCATTGAGGTGGCTCA	17	58.8	59.3
Gene expression		F-TCCATTTCCGAGCTGCAGAA	20	50.0	56.8
		R-GTGGAAGAAGTGCCTGCTGAT	21	52.4	57.6
Gene amplification	*BtMADS18*	F- ATGGGGAGAGGGCCGGTG	18	72.2	62.7
		R- GGAGCATCCATGGCGGAAGCTT	22	59.1	62.3
Gene expression		F- CACCAAGCACATCATCACCAAC ACC	25	52.0	60.5
		R- GAGCTTCTGACTCCCCTGATCCT CT	25	56.0	68.3
	BtMADS20	F- AGCTGCCTTCCTTCCCAAC	19	57.9	59.9
		R- CCACTGCTCGTCCGATCAA	19	57.9	59.8
	BteIF4α	F- ATGCTCTCCCGTGGTTTCAAG	21	52.4	57.5
		R- CAAGGGTAAGCTCATCTCTCTTCAC	25	48.0	56.9
Gene expression in transgenic plant	*BtMADS14*	F- GGCTAGTGCAATGGGACCAAA	21	52.4	60.9
		R- ATCTGAGGCTGCCCTGCTG	19	63.2	62.0
	BtFT1	F- TCCAAGCGACCCGAATCTT	19	52.6	59.0
		R- CTCGTAGCACATCACCTCTTG	21	52.4	58.5
	BtFT3	F- CCCAAGCGACCCGAATCTT	19	57.9	60.1
		R- CTCGTAGCACATCACCTCGTG	21	57.1	60.8
	AtFUL	F-AGAGGGAGAAGAAAACGGGTC	21	52.4	59.4
		R- CAACTCTCTCCACAAAGCCATC	22	50.0	59.3
	AtAP1	F- ACCAAATCCAGCATCCTTAC	20	45.0	62.0
		R- AGTTCGAGATCATTCCTCCTC	21	47.6	63.0
	AtACT2	F- AGTGGTCGTACAACCGGTATTGT	23	47.8	58.1
		R- GATGGCATGAGGAAGAGAGAAAC	23	47.8	55.5

3.3. Collection of plant tissues for nucleic acid isolation

Young, healthy leaves of *B. tulda* were collected to isolate genomic DNA. Total RNA was isolated from glume (G), lemma+palea+lodicule (LPL), stamen (ST) and carpel (C). Further, for seasonal and diurnal expression measurement of the target genes, total RNA was isolated from healthy young leaves in various seasons throughout the year and in various time points (8 am, 12 pm, 4 pm and 8 pm) in both long day and short day condition. For each of the tissue stage, tissues were collected from three biological replicates of *B. tulda* located in Shyamnagar, W.B. (SHYM7, SHYM16, 22.38° N. 88.40° E) and Bandel (BNDL22, 22.93° N. 88.38° E, Biswas et al., 2016). Similarly for RT-qPCR analyses, roots and flower tissues were also collected from wild type and transgenic Arabidopsis plants grown in long day condition in the laboratory. Tissues were immediately snap frozen in liquid nitrogen just after harvesting from field grown plants and kept in -80°C freezer for further use.

3.4. Isolation of genomic DNA, total RNA and cDNA library preparation

Frozen plant tissues were crushed in liquid N2 and then DNeasy Plant Mini Kit (Qiagen, Germany) was used to isolate genomic DNA following the established procedure. The combination of TRIzol and RNeasy method was employed to isolate total RNAs from collected tissues. The quantity and quality of the extracted DNA and RNA was checked by nanodrop and gel electrophoresis respectively.

For cDNA synthesis verso cDNA synthesis kit (THERMO SCIENTIFIC, USA) was used, approximately 1 μg of total RNA was used for each cDNA synthesis.

3.5. PCR amplification of the targeted *Bambusa tulda* genes

In order to perform the PCR amplification of the target genes from *B. tulda*, GoFlexi Taq DNA Polymerase (PROMEGA, USA) or ExTaq DNA Polymerase (TAKARA, USA) was used in S1000 PCR detection system. Reagents in the final PCR master mix were 5X buffer (GoFlexi Taq DNA Polymerase)/10X buffer (ExTaq DNA Polymerase), 1.5-3 mM MgCl2, 0.2 mM dNTPs, 0.25 μM forward and 0.25 μM reverse primers, 2 μL normalized cDNA/2ng/μL DNA and 1.25U/50 μL Taq polymerase. For GoFlexi Taq DNA Polymerase, PCR amplification cycle was: denaturation at 95ºC for 4 minutes, followed by 25-30 cycles of 94ºC for 30 seconds, annealing temperature for 30 seconds, 72ºC for 30-45 seconds for 1 kb of PCR product, final extension at 72ºC for 5 minutes, followed by 4ºC forever. For ExTaq DNA Polymerase, denaturation was done at 98ºC for 30 seconds, followed by 25-30 cycles of 98ºC for 10 seconds and so on. Amplified PCR products were visualized in 1.5% low EEO agarose (SRL, India) gel containing ethidium bromide under UV light exposure.

3.6. Gel elution of the amplified PCR products

The PCR product of desired molecular weight was purified using GeneJET gel elution kit (THERMO SCIENTIFIC, USA). For each amplified gene, ~120-180 μL PCR products were loaded on 2% agarose (SRL, India) gel stained with 10mg/ml ethidium bromide (C21H20BrN3, SRL, India). The DNA bands of desired molecular weight was dissolved into equal volume of binding buffer (THERMO SCIENTIFIC, USA) by incubating at 55-60ºC. The desired product was eluted in 20μl nuclease free water after subsequent washing and passing through DNA binding

column. The quantity and quality of the eluted DNA were checked in BioSpectrometer (Eppendorf, Germany) and 1.5% agarose (SRL, India) gel, respectively.

3.7. Preparation of calcium competent *E. coli* Dh5α cells

In order to perform cloning and transformation of the desired genes, calcium competent *E. coli* Dh5α cells were prepared. For this purpose, *E. coli* Dh5α cells were grown overnight as starter culture. On the next morning 250 ml of liquid LB medium was inoculated with 1 ml of starter culture and grown at 37 °C till the cell density reaches at 0.35-0.40 at 600 nm in a BioSpectrometer (Eppendorf, Germany). The cells were now spin down and successively treated with ice cold 100mM MgCl2, 100mM CaCl2 and 85mM CaCl2 in 15% glycerol respectively. The cells were snap frozen in 85mM CaCl2 in 15% glycerol with the help of liquid N2 and stored in -80 °C refrigerator for future use.

3.8. TA and gateway cloning of amplified PCR products and transformation

For TA cloning, approximately, 1:3 ratio of 25ng of vector DNA and the eluted PCR products were cloned into pGEM®-T Easy vector systems (Promega, USA). For cloning of the PCR products of length >2.5 kb, infusion HD cloning kit (Takara, USA) was used, where ~100 ng of eluted PCR product was cloned. Heat shock method was employed for transformation process. Blue-white screening and ampicillin sensitivity (100 µg/ml) were employed to identify putatively transformed bacterial colonies. For gateway cloning, the gateway specific tags were attached at the 5′ end of the *BtFT1*, *BtFT3* and *BtMADS14* specific primers and used for PCR amplification. Gel-eluted DNA fragments were used for cloning with pDONR221 donor vector (5:1 ratio) using the BP Clonase enzyme (INVITROGEN, USA). The transformation was done in *E. coli* DH5α competent cells and the isolated plasmids were sequenced to confirm correct insertion. Later, to perform LR reaction, pAlligator2 vector containing double CaMV35S promoter was used (Bensmihen et al., 2004). The expression clones were incorporated to competent *Agrobacterium tumefaciens* (pGV3101) by heat shock method.

3.9. Colony PCR

Ten to twenty single, white colonies were randomly picked with sterile plastic tips or wooden toothpicks from the transformed plate and added to individual PCR tubes containing 10 µL PCR

master mixture. The PCR amplifications were performed in S1000 PCR detection system with the above mentioned PCR cycling conditions except the initial denaturation step of 95ºC for 6 minutes to ensure complete disruption of bacterial cells and denaturation of DNA. The putatively transformed colonies were selected by exposing the gel containing amplified PCR products from each colony under UV-transilluminator.

3.10. Isolation of plasmids and Sanger's sequencing

The putatively transformed colonies identified from colony PCR were grown overnight at 37º C in 10 ml liquid LB medium with selectable marker. On the next morning, plasmids were isolated using GeneJET Plasmid Miniprep kit (THERMO SCIENTIFIC, USA). The column bound plasmids were eluted in 30µl nuclease free water and were sent for Sanger's sequencing either in AgriGenome Labs (http://www.aggenome.com/) or Barcode Biosciences Pvt. Ltd. (https://www.barcodebiosciences.com/). The trimmed nucleotide sequences obtained after Sanger's sequencing were assembled in the CAP3 program (http://doua.prabi.fr/ software/cap3) and were finally deposited to NCBI database (http://www.ncbi.nlm.nih.gov/).

3.11. Phylogeny, bubble plot and protein subcellular localization analyses

The expansion or reduction of gene numbers were noted for each member of BOP clade and was visualized as bubble plot using Origin 2024 software. MEGA11 software was employed to analyze phylogenetic relationship of deduced amino acid sequences of coding regions obtained from bamboos and its phylogenetic neighbours. A Neighbour Joining (NJ) tree was built using the bootstrap values for 1000 replicates. The subcellular localizations of the proteins were predicted by WoLF PSORT (https://www.genscript.com/tools/wolf-psort).

3.12. Synteny analyses

The synteny analysis was performed comparing the *P. edulis* genome against rice and Brachypodium. Among all the sequenced bamboos, the best genome coverage was found for *P. edulis* genome due to use of multiple sequencing strategies and quality of the annotation and the chromosome level assembly is also present (Zhao et al. 2018). Total 24 scaffolds were obtained from the genome annotation file and each corresponds to an individual chromosome. The target protein sequences were aligned using the TBtools software (https://bio.tools/tbtools). The default

parameters of the MCScanX algorithm were initially used to infer the syntenic relationships and the duplication events of the protein sequences in a pairwise manner, i.e., *P. edulis* vs. rice and *P. edulis* vs. *Brachypodium*. Further, the same algorithm was again used to study the collated syntenic relationship among the three species and the results were visualized using Multiple Synteny Plot in TBtools software.

3.13. *In silico* characterization of amino acid sequences

From the obtained CDS sequences of the target genes, first the putative amino acid sequences were predicted in the ExPASy translate tool (https://www.expasy.org/) and then the amino acid sequences were multiple aligned with the homologous sequences from other closely related monocotyledonous plants in the MUSCLE program (https://www.ebi.ac.uk/jdispatcher/msa/muscle) and functional domains and biological important residues were identified. The molecular weight of the protein, isoelectric point, number of positively and negatively charged residues, aliphatic index, instability index and grand average of hydropathicity (GRAVY) were calculated using ExPASy database (https://www.expasy.org/).

3.14. Real time RT-qPCR to analyze gene expression and statistical analyses

The real time RT-qPCR experiments were performed with the SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, USA) in a CFX connect real-time PCR detection system (BIO-RAD, USA). Melt curve analysis was performed to avoid primer dimers formation. The *B. tulda Eukaryotic Initiation Factor 4α* (*BteIF4α*) and *A. thaliana Actin2* (*AtACT2*) genes were used as reference to normalize expression data of bamboo (Chakraborty et al., 2019) and Arabidopsis (Das et al., 2016), respectively. $2^{-\Delta\Delta C_T}$ method was used to calculate the relative fold changes (Livak and Schmittgen, 2001). The root tissue was used as calibrator since this is not related to flowering. The difference in the expression fold changes for the transgenic plants compared to WT plants were statistically verified by a two-sample t-test.

3.15. Expression analyses, heat map and gene regulatory network of *Phyllostachys edulis MADS-box* genes

Public availability of transcriptome data for bamboo is very limited. Moreover, inclusion of insufficient biological replicates or incomplete tissue descriptions posed additional challenges to

include many transcriptome libraries. Eventually, one Illumina Hiseq 2500 transcriptome library of *P. edulis* (Accession: PRJNA496390; GEO: GSE121216, Zhang et al., 2021a) obtained from NCBI database were used for the expression analyses. This transcriptome library includes five tissues with three biological replicates for each (leaves from three weeks old seedlings, leaves from one year old plants, leaves from the plants those will flower next year, leaves from flowering plants and flower florets). Heat map was generated using Heatmapper (Babicki et al., 2016) with average tpm values (three replicates). Clustering was done using Pearson correlation and average linkage.

In order to predict the putative flower related functions of *P. edulis MADS-box* homologs, gene regulatory network analyses were performed. *P. edulis* genes, which were homologous to rice were identified by phylogenetic analyses and were then mapped against the gene regulatory network known in rice (Hu et al., 2015).

3.16. *In planta* transformation, GFP screening, phenotypic characterization of the *Bambusa tulda FT1, FT3* and *MADS14* overexpressed *Arabidopsis* plants

For the *Agrobacterium* mediated transformation of the *BtFT1*, *BtFT3* and *BtMADS14*, approximately 6 weeks old, healthy *A. thaliana* Col-0 plants were selected for floral dip method (Clough and Bent, 1998). The strong green fluorescence of the GFP reporter gene of putatively transgenic seeds were observed under fluorescent microscope (Nikon Eclipse Ti2) and selected (Bensmihen et al., 2004). The WT and transgenic plants were grown in long day (LD, 16-h light and 8-h dark) and their bolting time was recorded. In addition, number of the rosette leaves were counted from 30 independent transgenic plants of T3 generation along with Col-0 at an interval of 4 days from 10 days after germination (DAG) until 30 days. The one-way analysis of variance (ANOVA) was carried out to test if the difference in rosette leaf number is statistically significant at $p \leq 0.05$. A two-sample t-test was performed to investigate whether the bolting time and seed numbers per individual silique of transgenic plants were significantly different from the WT plants. All the statistical analyses were performed in IBM SPSS Statistics Version 20.

4. Results

- **Objective 1**

4.1. *In silico* identification and phylogenetic analysis of important vernalization

pathway gene homologs from sequenced bamboo genome

*VIL*s are important genes involved in vernalization pathway in all major Pooids including bamboo. However, the bamboo *Ghd7* is the functional homolog of *VRN2* (wheat, barley, Brachypodium) and *CO9* (Barley) present in other Pooids. Total 81 *VIL* and 27 *Ghd7* gene homologs have been identified in 11 sequenced bamboos by using rice genes as query (**Table 2**).

Table 2. List of homologous vernalization pathway genes identified in sequenced bamboos.

Gene	Plant name	Gene ID	Gene name	Identity (%)	E value	Length of amino acid	Database
VIL1	*Oryza sativa*	LOC_Os12g34850	*OsVIL1*	-	-	707	https://phytozome-next.jgi.doe.gov/
	Brachypodium distachyon	Bradi4g05950	*BdVIL1*	75	0	696	https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/
	Olyra latifolia	Ol10g09270.mRNA1	*OlVIL1*	80	0	716	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0031565.3	*RgVIL1*	79	0	702	
	Ampelocalamus luodianensis	Alu12Cg04510.1	*AlVIL1-1*	80	0	699	https://bamboo.genobank.org/
		Alu12Dg06690.2	*AlVIL1-2*	81	0	699	
	Hsuehochloa calcarea	Hca12Cg05590.2	*HcVIL1-1*	80	0	699	
		Hca12Dg05770.2	*HcVIL1-2*	79	0	698	
	Phyllostachys edulis	PH02Gene00673.t1	*PeVIL1-1*	80	0	699	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		PH02Gene27685.t1	*PeVIL1-2*	81	0	699	
	Guadua angustifolia	GanEVM0018849.2	*GaVIL1-1*	81	0	711	
		GanEVM0036937.2	*GaVIL1-2*	81	0	699	
	Otatea glauca	Ogl12Bg06080.1	*OgVIL1-1*	80	0	698	https://bamboo.genobank.org/
		Ogl03Cg09950.1	*OgVIL1-2*	81	0	699	
	Rhipidocladum racemiflorum	Rhi12Bg03950.1	*RrVIL1*	81	0	698	
	Bambusa tulda	MN782514	*BtVIL1*	82	0	656	https://www.ncbi.nlm.nih.gov/
	Bonia amplexicaulis	Bam12Bg02820.mRNA1	*BaVIL1-1*	81	0	708	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		Bam03Cg09710.mRNA1	*BaVIL1-2*	81	0	702	
	Melocanna baccifera	Mhu12Bg05010.2	*MbVIL1-1*	81	0	698	https://bamboo.genobank.org/
		Mhu03Cg07430.1	*MbVIL1-2*	80	0	702	
		Mhu12Ag02830.1	*MbVIL1-3*	80	0	703	
	Dendrocalamus sinicus	Dsi12Bg05010.1	*DsVIL1-1*	81	0	698	
		Dsi12Bg05000.1	*DsVIL1-2*	71	0	636	
		Dsi03Cg08040.1	*DsVIL1-3*	82	0	702	
		Dsi12Ag03230.1	*DsVIL1-4*	80	0	699	
VIL2	*Oryza sativa*	LOC_Os02g05840	*OsVIL2*	-	-	749	https://phytozome-next.jgi.doe.gov/
	Brachypodium distachyon	Bradi3g04140	*BdVIL2-1*	78	0	754	https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/
		Bradi1g33450	*BdVIL2-2*	56	0	524	
	Olyra latifolia	Ol05g29700.mRNA1	*OlVIL2*	86	0	755	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0006661.1	*RgVIL2*	84	0	753	
	Ampelocalamus luodianensis	Alu02Cg03510.2	*AlVIL2-1*	86	0	754	https://bamboo.genobank.org/
		Alu02Dg24620.1	*AlVIL2-2*	87	0	745	
	Hsuehochloa calcarea	Hca02Cg02990.3	*HcVIL2-1*	85	0	754	
		Hca02Dg02990.1	*HcVIL2-2*	87	0	748	

	Phyllostachys edulis	PH02Gene24739.t2	*PeVIL2-1*	86	0	754	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		PH02Gene26055.t1	*PeVIL2-2*	86	0	745	
		PH02Gene02714.t1	*PeVIL2-3*	61	0	864	
	Guadua angustifolia	GanEVM0023398.2	*GaVIL2*	86	0	754	
	Otatea glauca	Ogl02Bg02430.1	*OgVIL2-1*	86	0	750	https://bamboo.genobank.org/
		Ogl02Cg07470.2	*OgVIL2-2*	86	0	754	
		Ogl06Bg14990.1	*OgVIL2-3*	56	0	680	
	Rhipidocladum racemiflorum	Rhi02Cg16700.1	*RrVIL2-1*	85	0	750	
		Rhi02Bg22400.1	*RrVIL2-2*	86	0	751	
	Bambusa tulda	PP663281	*BtVIL2*	87	0	754	https://www.ncbi.nlm.nih.gov/
	Bonia amplexicaulis	Bam02Cg09740.mRNA1	*BaVIL2-1*	86	0	749	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		Bam02Bg01810.mRNA1	*BaVIL2-2*	87	0	756	
		Bam02Ag01860.mRNA1	*BaVIL2-3*	85	0	755	
	Melocanna baccifera	Mhu02Cg10900.2	*MbVIL2-1*	85	0	740	https://bamboo.genobank.org/
		Mhu02Bg01770.1	*MbVIL2-2*	86	0	790	
		Mhu02Ag17480.2	*MbVIL2-3*	86	0	750	
	Dendrocalamus sinicus	Dsi02Bg01950.2	*DsVIL2-1*	87	0	756	
		Dsi02Ag01950.1	*DsVIL2-2*	86	0	754	
VIL3	*Oryza sativa*	LOC_Os05g05310	*OsVIL3*	-	-	607	https://phytozome-next.jgi.doe.gov/
	Brachypodium distachyon	Bradi2g36237	*BdVIL3*	73	0	611	https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/
	Olyra latifolia	Ol05g08700.mRNA1	*OlVIL3*	79	0	612	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0022324.1	*RgVIL3*	76	0	620	
	Ampelocalamus luodianensis	Alu09Cg20910.1	*AlVIL3-1*	81	0	613	https://bamboo.genobank.org/
		Alu05Dg16790.1	*AlVIL3-2*	80	0	613	
	Hsuehochloa calcarea	Hca09Cg02480.2	*HcVIL3-1*	80	0	613	
		Hca05Dg13930.1	*HcVIL3-2*	76	0	563	
	Phyllostachys edulis	PH02Gene33036.t1	*PeVIL3-1*	81	0	613	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		PH02Gene17384.t1	*PeVIL3-2*	81	0	613	
	Guadua angustifolia	GanEVM0040100.7	*GaVIL3-1*	80	0	612	
		GanEVM0008949.1	*GaVIL3-2*	80	0	610	
	Otatea glauca	Ogl05Cg03290.2	*OgVIL3-1*	80	0	608	https://bamboo.genobank.org/
		Ogl05Bg02240.1	*OgVIL3-2*	80	0	610	
	Rhipidocladum racemiflorum	Rhi05Cg12450.1	*RrVIL3-1*	80	0	608	
		Rhi05Bg14550.1	*RrVIL3-2*	77	0	609	
	Bambusa tulda	Btu05C.g00266t22	*BtVIL3*	79	0	612	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Bonia amplexicaulis	Bam05Cg02190.mRNA1	*BaVIL3*	79	0	612	
	Melocanna baccifera	Mhu05Cg02160.2	*MbVIL3-1*	79	0	611	https://bamboo.genobank.org/
		Mhu05Ag01440.1	*MbVIL3-2*	81	0	613	
		Mhu05Bg11990.1	*MbVIL3-3*	80	0	609	
	Dendrocalamus sinicus	Dsi05Cg10510.1	*DsVIL3-1*	79	0	612	
		Dsi05Ag01570.2	*DsVIL3-2*	81	0	613	
VIL4	*Oryza sativa*	LOC_Os08g12430	*OsVIL4*	-	-	630	https://phytozome-next.jgi.doe.gov/
	Brachypodium distachyon	Bradi3g17950	*BdVIL4*	73	0	631	https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/
	Olyra latifolia	Ol08g22750.mRNA1	*OlVIL4*	77	0	625	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0012641.1	*RgVIL4*	78	0	638	
	Ampelocalamus luodianensis	Alu08Cg12160.1	*AlVIL4*	79	0	623	https://bamboo.genobank.org/
	Hsuehochloa calcarea	Hca08Dg03770.2	*HcVIL4-1*	79	0	642	
		Hca08Cg10400.1	*HcVIL4-2*	80	0	636	
	Phyllostachys edulis	PH02Gene39446.t1	*PeVIL4-1*	80	0	654	https://gendbe.computational.bio.uni-
		PH02Gene16610.t1	*PeVIL4-2*	79	0	669	

	Guadua angustifolia	GanEVM0007915.2	*GaVIL4-1*	79	0	631	giessen.de/cgi-bin/blast_interface.cgi
		GanEVM0003171.3	*GaVIL4-2*	79	0	654	
	Otatea glauca	Ogl08Bg04050.2	*OgVIL4-1*	79	0	630	https://bamboo.genobank.org/
		Ogl07Cg15040.1	*OgVIL4-2*	80	0	631	
	Rhipidocladum racemiflorum	Rhi08Bg04620.1	*RrVIL4-1*	79	0	632	
		Rhi07Cg15130.1	*RrVIL4-2*	79	0	640	
	Bambusa tulda	MT013336	*BtVIL4*	79	0	630	https://www.ncbi.nlm.nih.gov/
	Melocanna baccifera	Mhu08Bg07980.1	*MbVIL4*	79	0	653	https://bamboo.genobank.org/
	Dendrocalamus sinicus	Dsi08Bg03540.1	*DsVIL4-1*	79	0	653	
		Dsi07Cg00380.1	*DsVIL4-2*	80	0	642	
Ghd7	*Oryza sativa*	LOC_Os07g15770	*OsGhd7*	-	-		https://phytozome-next.jgi.doe.gov/
	Olyra latifolia	Ol10g00900.mRNA1	*OlGhd7*	48	4e-43		https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0027149.1	*RgGhd7*	49	4e-41	257	
	Ampelocalamus luodianensis	Alu03Cg32430.1	*AlGhd7-1*	55	2e-56	233	https://bamboo.genobank.org/
		Alu10Dg01300.2	*AlGhd7-2*	51	9e-28	271	
		Alu10Cg11100.2	*AlGhd7-3*	54	2e-38	250	
	Hsuehochloa calcarea	Hca03Cg30540.2	*HcGhd7*	56	9e-50	232	
	Phyllostachys edulis	PH02Gene36002.t1	*PeGhd7*	45	6e-32	207	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Guadua angustifolia	GanEVM0025404.1	*GaGhd7-1*	49	3e-51	247	
		GanEVM0006673.1	*GaGhd7-2*	51	4e-40	263	
		GanEVM0042441.1	*GaGhd7-3*	47	1e-38	272	
	Otatea glauca	Ogl03Bg00060.1	*OgGhd7-1*	52	2e-59	245	https://bamboo.genobank.org/
		Ogl10Cg03600.1	*OgGhd7-2*	58	2e-78	280	
		Ogl10Bg00940.1	*OgGhd7-3*	55	5e-33	270	
	Rhipidocladum racemiflorum	Rhi10Cg09000.1	*RrGhd7*	60	4e-83	266	
	Bambusa tulda	Btu10B.g00866t48	*BtGhd7*	54	8e-35	265	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Bonia amplexicaulis	Bam01Ag10220.mRNA1	*BaGhd7-1*	50	7e-46	231	
		Bam10Cg00080.mRNA1	*BaGhd7-2*	50	4e-45	237	
		Bam10Cg02670.mRNA1	*BaGhd7-3*	53	7e-31	275	
		BamCtg01490g00010.mRNA1	*BaGhd7-4*	45	6e-24	244	
		Bam10Bg06810.mRNA1	*BaGhd7-5*	48	3e-34	239	
	Melocanna baccifera	MhuCtg00087g00040.1	*MbGhd7-1*	52	4e-39	286	https://bamboo.genobank.org/
		Mhu10Bg00940.2	*MbGhd7-2*	59	8e-38	267	
		Mhu10Cg02590.2	*MbGhd7-3*	52	4e-34	277	
		Mhu10Ag00680.2	*MbGhd7-4*	57	2e-67	242	
	Dendrocalamus sinicus	Dsi01Ag11080.1	*DsGhd7-1*	51	6e-48	243	
		DsiCtg00186g00060.1	*DsGhd7-2*	50	1e-47	245	
		Dsi10Cg06880.1	*DsGhd7-3*	53	1e-30	275	
		Dsi10Ag00810.1	*DsGhd7-4*	52	7e-37	256	
		Dsi10Bg08360.1	*DsGhd7-5*	54	4e-34	260	

Single copies of each of the *VIL* and *Ghd7* genes have been identified from the tropical bamboo *B. tulda* by Sanger's sequencing. At least one copy of *VIL1*, *VIL2*, *VIL3* and *Ghd7* could be identified in each of the bamboo species studied, except for *B. amplexicaulis*, which lacked the *VIL4* gene (**Figure 4**). Among all *VILs*, *D. sinicus* contained highest number of of *VIL1* gene copies (4). The hexaploid *M. baccifera* contained 3 copies for each of the *VIL1*, *VIL2* and *VIL3* genes in contrast to single copy gene present in rice. In case of *Ghd7*, all the hexaploid sequenced bamboo members such as *B. amplexicaulis* (5), *M. baccifera* (4) and *D. sinicus* (5) contained higher number of homologs (1, **Figure 4**).

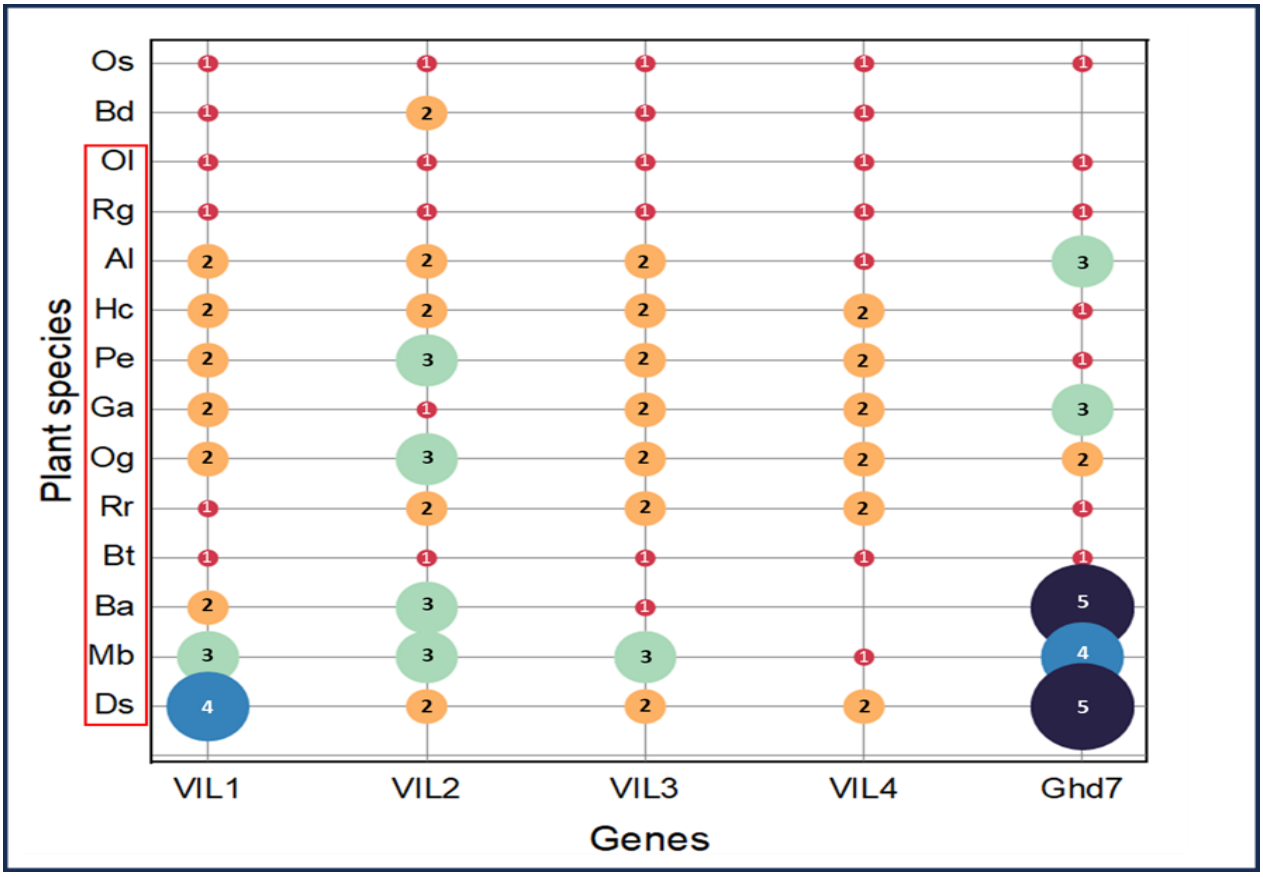

Figure 4. Bubble plot to demonstrate differential numbers of *VIL* and *Ghd7* genes identified in 12 studied bamboo species, rice and *Brachypodium*. The red rectangle marks all bamboo species.

Abbreviations used: Os- *Oryza sativa*, Bd- *Brachypodium distachyon*, Ol- *Olyra latifolia*, Rg- *Raddia guianensis*, Al- *Ampelocalamus luodianensis*, Hc- *Hsuehochloa calcarea*, Pe- *Phyllostachys edulis*, Ga- *Guadua angustifolia*, Og- *Otatea glauca*, Rr- *Rhipidocladum racemiflorum*, Bt- *Bambusa tulda*, Ba- *Bonia amplexicaulis*, Mb- *Melocanna baccifera*, Ds- *Dendrocalamus sinicus*.

Phylogenetic analysis revealed clustering of all *VIL* homologs in 4 distinct clusters corresponding to *VIL1*, *VIL2*, *VIL3* and *VIL4* clades (**Figure 5**). Among them, *VIL2* was the largest group containing total 27 homologs. *VIL1* is the second largest cluster with 25 homologous members (**Figure 5**). The clustering of the 4 groups revealed the closeness between *VIL1*/*VIL3* and *VIL2*/*VIL4* indicating shared ancestral origin.

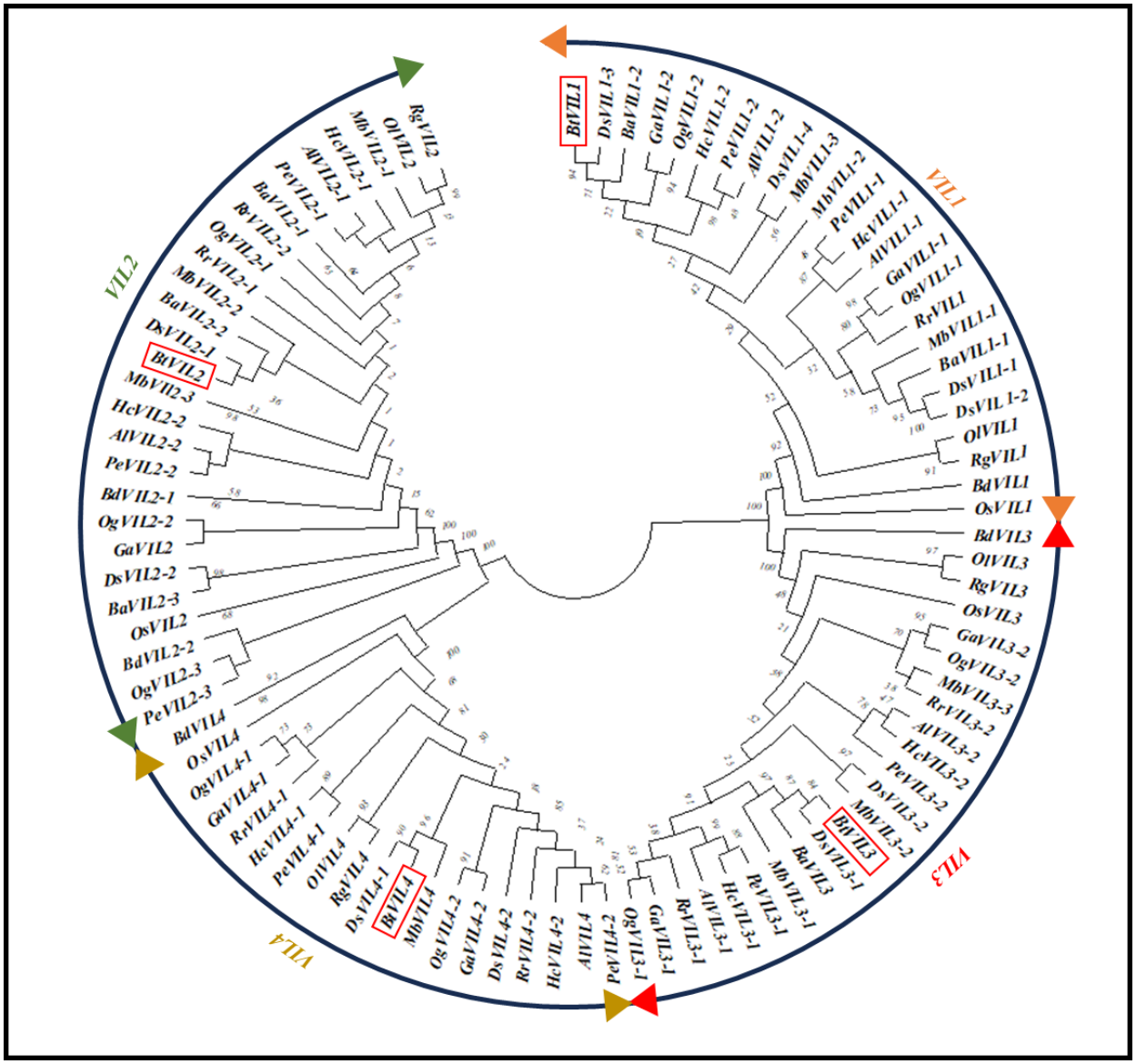

Figure 5. Phylogenetic tree of *VIL* genes using selected bamboo and related Poaceae members. Genes identified by targeted sequencing in *B. tulda* have been highlighted in red boxes.

Total 29 *Ghd7* genes obtained from selected members of Poaceae have been considered for studying phylogenetic relationship. *Brachypodium* does not contain any *Ghd7* homolog, while *B. amplexicaulis* and *D. sinicus* represented highest numbers of *Ghd7* genes (5 in each). The phylogenetic clustering of the *Ghd7* genes show the out grouping of rice *Ghd7* and all the bamboo *Ghd7* genes were clustered together due to their sequence similarity.

4.2. *In silico* characterization of putative VIL and Ghd7 proteins in bamboo

Amino acids sequence of predicted bamboo VIL proteins vary greatly with respect to many important parameters. For instance, VIL2 was the longest protein and Ghd7 was the shortest one. The PeVIL2-3 was the longest protein among all by having 864 amino acids and approximate molecular weight (MW) 94826.47 KDa. The PeGhd7 was the shortest protein with 207 amino acids and MW 23053.04 KDa. All the VIL and Ghd7 proteins have negative grand average of hydropathicity (GRAVY) indicating their possible hydrophilic nature.

4.3. Conservation of domains and important amino acids in bamboo VIL and Ghd7 protein sequences

All bamboo VIL protein sequences have 3 functionally important domains, which are plant homeodomain finger (PHD), fibronectin type 3 domain (FN3) and *VIN3*-interacting domain (VID). *In silico* study on amino acid sequences of bamboo VIL1 (**Figure 6**), VIL2, VIL3 and VIL4 showed conservation of these domains. However, in a few sequences, loss of stretch of aminoc acids have been noticed. For instance, the DsVIL1-2 lacked 10 amino acids and one of them were involved in C4HC3 motif, which was a part of PHD domain involved in epigenetic modification of the target gene. Similarly, 12 amino acids were missing from the VID domain of DsVIL1-2 (**Figure 6**). The BtVIL1 contained all the domains intact indicating its possible functional conservation. Similar observations have been made on BtVIL2, BtVIL3, BtVIL4.

Figure 6. Multiple sequence alignment of amino acid sequences of VIL1 proteins A. PHD domain B. FN3 domain C. VID domain. The red star indicates amino acids involved in C4HC3 finger motif formation. BtVIL1 marked in red font has been sequenced by targeted sequencing.

The Ghd7 proteins have CCT (*CO*, *CO-like* and *TOC1*) domain, which is involved in the protein-protein interaction and nuclear localization, thus helping in transcriptional regulation. Multiple sequence alignment of CCT domains of the bamboo Ghd7 proteins reveal presence of full length

domain without any sequence change (**Figure 7**).

Figure 7. Multiple sequence alignment of CCT domains identified in Ghd7 proteins. The red star indicates amino acids needed to maintain the protein function. BtGhd7 marked in red font has been sequenced by targeted sequencing.

4.4. Syntenic relationship among *Phyllostachys edulis*, rice and *Brachypodium VIL* and *Ghd7* genes

Rice and *Brachypodium* belongs to Bambusoideae Oryzoideae Pooideae (BOP) clade of the family Poaceae. Both of them are phylogenetically close to bamboo and have well assembled, annotated genome with good coverage. Thus, both the genomes offer good prospects to carry out evolutionary analysis including synteny.

The 4 *VIL* gene copies in rice are distributed on chromosomes 2, 5, 8 and 12. Five *VIL* gene copies of *Brachypodium* are distributed on Chr1, Chr2, Chr3 and Chr4 (**Figure 8**). Total 9 copies of *VIL* genes have been identified in *P. edulis* against 4 rice *VIL* genes. The *P. edulis VIL* are distributed on scaffolds 8, 9, 11, 12, 13, 17, 18, 20 and 22.

Comparison between *P. edulis* and rice revealed many-to-one syntenic relationship for *PeVIL1-1-PeVIL1-2*: *OsVIL1*, *PeVIL2-1-PeVIL2-2-PeVIL2-3*: *OsVIL2*, *PeVIL3-1-PeVIL3-2*: *OsVIL3* and

PeVIL4-1-PeVIL4-2: *OsVIL4* (**Figure 8**). Similarly, comparison between *P. edulis* and Brachypodium revealed many-to-one syntenic relationship for *PeVIL1-1-PeVIL1-2*: *BdVIL1*, *PeVIL3-1-PeVIL3-2*: *BdVIL3* and *PeVIL4-1-PeVIL4-2*: *BdVIL4*. Many-to-many syntenic relationship was found for *PeVIL2-1-PeVIL2-2-PeVIL2-3*: *BdVIL2-1-BdVIL2-2* (**Figure 8**).

In case of *Ghd7*, *Brachypodium* has no homologous member. There is a single copy of *Ghd7* in *P. edulis* distributed on scaffold 15, while rice *Ghd7* is located on Chr10.

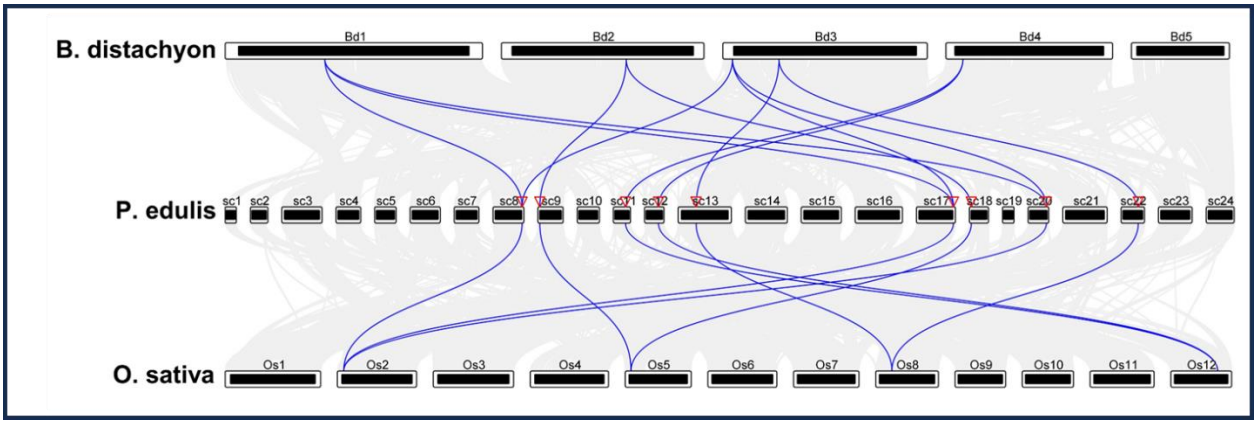

Figure 8. Synteny analysis of *VIL* genes among *Phyllosatchys edulis*, *Brachypodium distachyon* and *Oryza sativa*. The grey lines in the background indicate collinear blocks of *P. edulis* with *B. distachyon* and *O. sativa* genomes. The blue lines highlight the syntenic gene pairs.

4.5. Seasonal and diurnal expression analyses of selected vernalization pathway genes and their potential transcriptional targets in *Bambusa tulda*

Since vernalization pathway is responsive to low temperature, in order to study the impact of cold regulation on selected vernalization pathway genes before and during flowering, expressions of these genes were studied in selected seasons having considerable temperature fluctuations. The expression analyses were performed both in young leaf from flowering plants (YLF) and young leaf from non-flowering plants (YLN) in 4 different months (June, October, January, March) representing different seasons: summer autumn, winter and spring). This study was conducted on a sporadic flowering population of bamboo where flowering occurred in spring. All the *B. tulda VIL* genes (*BtVIL1*, *BtVIL2*, *BtVIL3*, *BtVIL4*) demonstrated elevated expression in winter both for YLF (**Figure 9A**) and YLN (**Figure 9B**), but the extent of transcriptional alteration was much higher in the YLF than YLN indicating their possible roles in flower induction.

The expression levels of target genes of *VIL*s such as *Ghd7* and *LFL1* were also evaluated in all

the tissues. It was previously known that in summer, *VIL* induces *Ghd7* thus repressing flowering and in winter, *VIL* suppresses *LFL1,* thereby, activation of *Ehd1* happens inducing flowering. In the YLF tissues, the above-mentioned trend can be observed (**Figure 9C**) but not in YLN (**Figure 9D**), further proving the maintenance of flowering pathway known in other plants.

All the signals coming from vernalization pathway ultimately integrates through *FT* to induce flowering. In *B. tulda*, 4 copies of *FT* have been identified previously (*BtFT1, BtFT2, BtFT3, BtFT4*, Dutta et al., 2018). The diurnal expression pattern of these genes demonstrated the involvement of *BtFT3* and *BtFT4* in flowering. Therefore, the seasonal expression pattern of *BtFT3*/*BtFT4* was studied. The elevated expression in the YLF (**Figure 9E**) but not in YLN (**Figure 9F**) during winter proves the integration of vernalization pathway gene signals and involvement of *FT* gene in flowering.

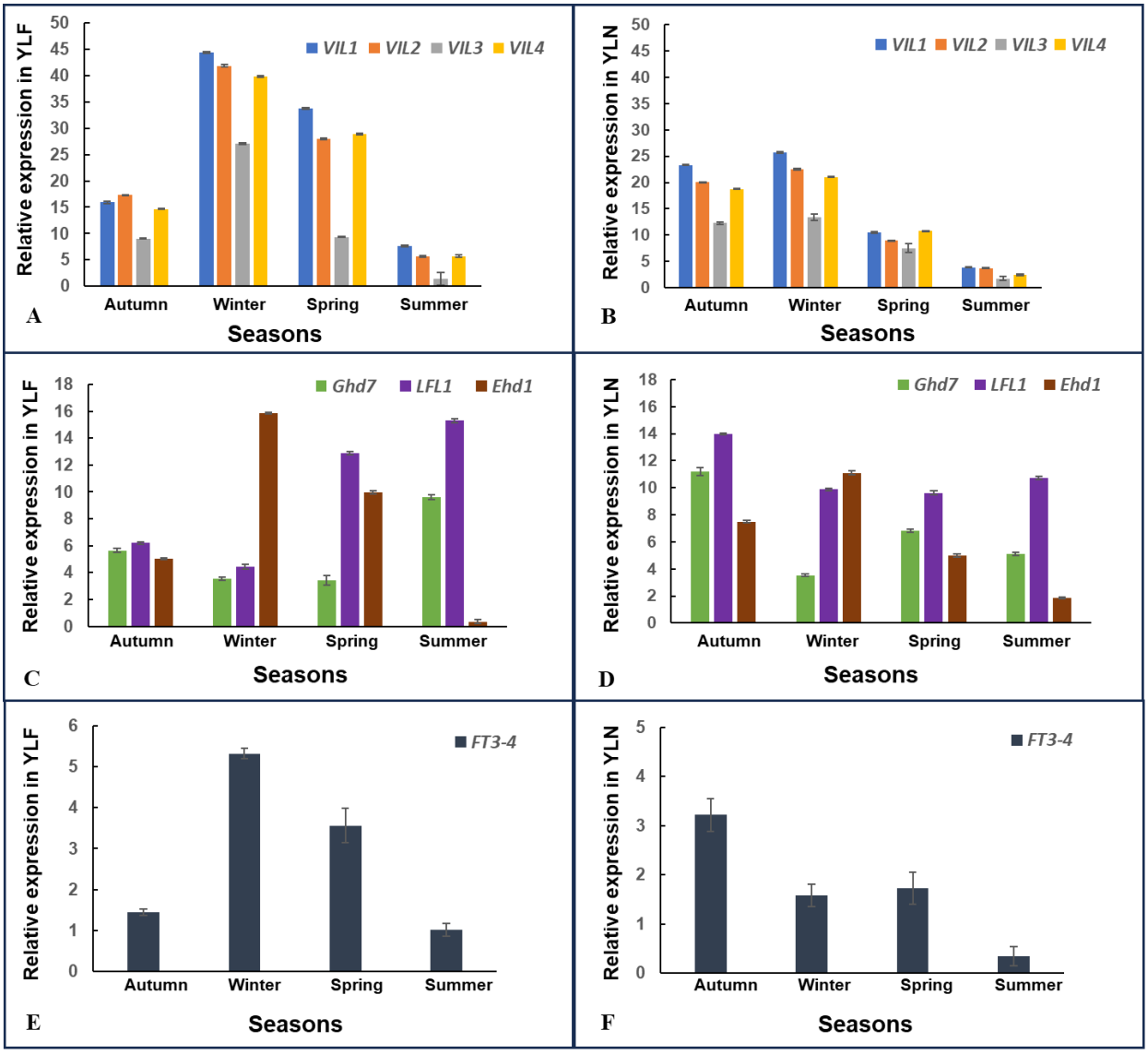

Figure 9. Study of expression levels of (A) *BtVIL1, BtVIL2, BtVIL3, BtVIL4* in YLF (B) *BtVIL1, BtVIL2, BtVIL3, BtVIL4* in YLN (C) *BtGhd7, BtLFL1, BtEhd1* in YLF (D) *BtGhd7, BtLFL1, BtEhd1* in YLN (E) *BtFT3-4* in YLF (F) *BtFT3-4* in YLN in different seasons throughout the year. Transcript expression of eIF4α was used to normalize expression data of the targeted flowering genes. The relative fold change was calculated by $2^{-\Delta\Delta C_T}$ method using the expression level observed in rhizome as the calibrator. Each bar represents mean of three biological replicates±SE. Abbreviations used: YLF- young leaf from flowering plants, YLN- young leaf from non-flowering plants.

It is previously known in rice that vernalization pathway genes and their transcriptional targets are also involved in the photoperiodic pathway of flowering. Thus, to study the diurnal responses of

bamboo *VIL* genes and to assess their comparability to that of rice homologs, RT-qPCR analyses of *VIL*s were carried out both in YLF and YLN at different time points of a day in long day (LD, **Figure 10A, B**) and short day (SD, **Figure 10C, D**). The expression analyses of the transcriptional target genes were also carried out in YLF and YLN in LD (**Figure 10E, F**) as well as SD (**Figure 10G, H**).

The expression changes of *VIL2* and *VIL3* in YLF was most apparent at night (8pm) in SD (**Figure 10C**). In the same tissues, *VIL1* and *VIL4* expressions elevate after 12 noon. All the genes show lowest expression at 12 noon. In the SD YLN, the gene expression pattern is more or less opposite except for the *VIL3* (**Figure 10D**), proving it's another role except flowering. In the SD YLF, the diurnal pattern of the *Ghd7* and *LFL1* show suppression by *VIL* genes (**Figure 10G**). The suppression of *LFL1* by *VIL* at night induces *Ehd1*, thereby promoting flowering. In the SD YLN, almost contrast expression patterns of these target genes are noted (**Figure 10H**). Hence, from the above experiment, the involvement of the vernalization pathway genes in photoperiodic pathway could be proved.

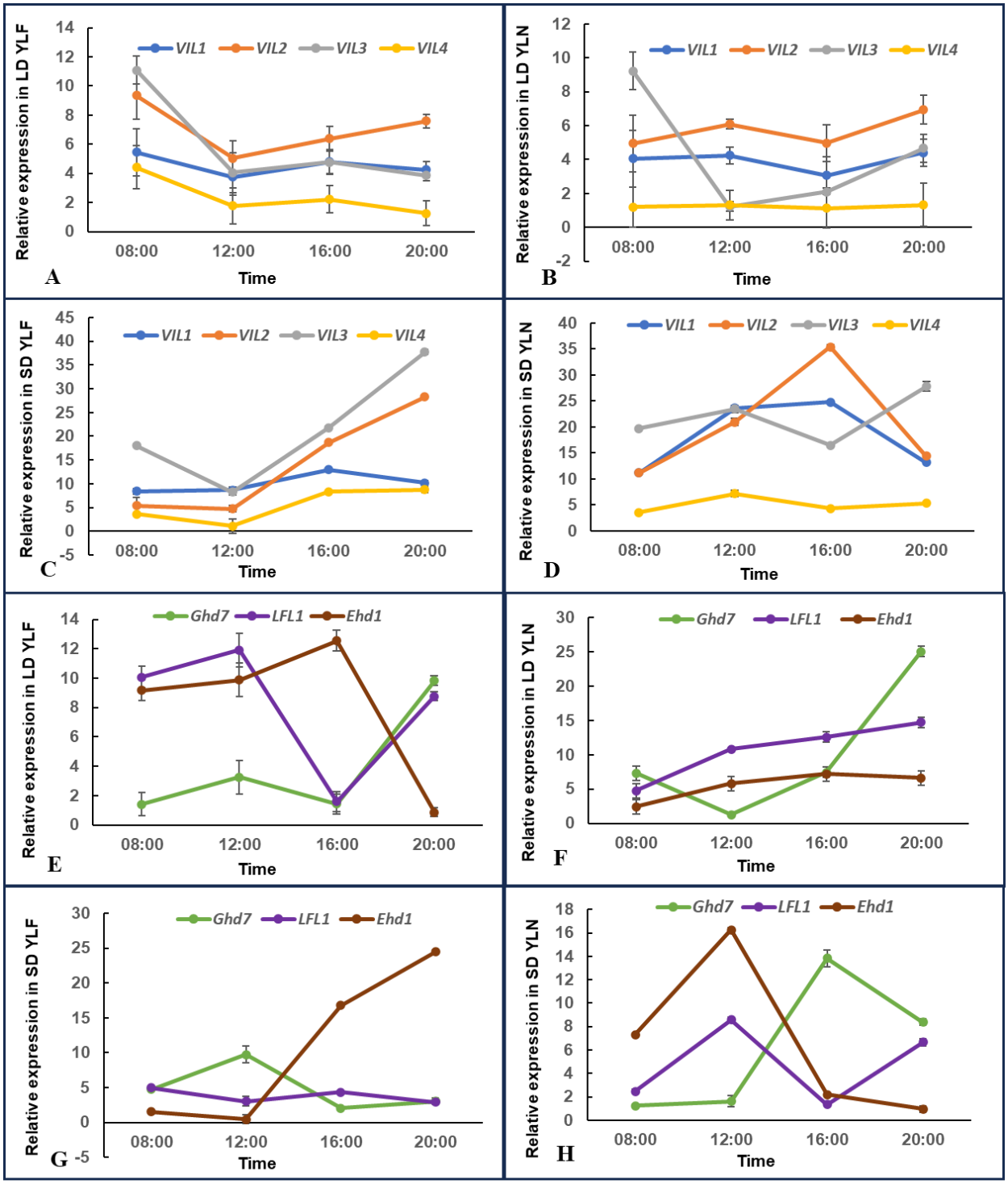

Figure 10. Study of diurnal expression levels of *BtVIL1, BtVIL2, BtVIL3, BtVIL4* in (A) LD YLF (B) LD YLN (C) SD YLF (D) SD YLN. Diurnal expression levels of *BtGhd7, BtLFL1, BtEhd1* in (E) LD YLF (F) LD YLN (G) SD YLF (H) SD YLN. Transcript expression of

eIF4α was used to normalize expression data of the targeted flowering genes. The relative fold change was calculated by $2^{-\Delta\Delta C_T}$ method using the expression level observed in rhizome as the calibrator. Each bar represents mean of three biological replicates±SE. Abbreviations used: LD- long day, SD- short day, YLF- young leaf from flowering plants, YLN- young leaf from non-flowering plants.

❖ **Objective 2**

4.6. Identification and copy number comparison of *MADS-box* genes in bamboos

A total of 701 *MADS-box* genes that included all type I and II could be obtained from *Brachypodium* and 11 sequenced bamboos in BLAST search against 73 rice *MADS-box* genes. Though there is a report of presence of 75 *MADS-box* genes in rice (Arora et al., 2007), sequence data could be found for only 73 (https://phytozome.jgi.doe.gov/pz/portal.html). Among 701 *MADS-box* genes, 663 were found in 11 bamboo species which were subjected to whole genome sequencing, while 4 copies of *APETALA1* genes were identified from tropical bamboo *B. tulda* by Sanger's sequencing. The type I *MADS-box* genes included *Mα*, *Mβ* and *Mγ* subtypes and type II included *MIKC**, *AG-like*, *AGL2-like*, *AGL-6-like*, *AGL12-like*, *AGL17-like*, *AP1/SQUA-like*, *DEF-like*, *GGM13-like*, *GLO-like*, *OsMADS32-like*, *StMADS11-like* and *TM3-like* genes.

Among all bamboos, the highest number of *MADS-box* genes could be found in the hexaploid bamboo *M. baccifera* and *D. sinicus* (82 in each, **Figure 11**), while lowest was in the diploid bamboo *R. guianensis* (33, **Figure 11**). This could be a direct consequence of polyploidization. Approximately 27% of all rice *MADS-box* genes were of type I, which was much less in bamboos ranging from 11% (*A. luodianensis*) to 19% (*H. calcarea* and *B. amplexicaulis*). The type II *MADS-box* genes that regulate flower development constitute approximately 67% of all *MADS-box* genes, which was in a range of 73% (*H. calcarea*, *P. edulis*) to 81% (*R. guianensis*) in case of bamboos. Particularly, the *AG-like* (72), *AGL2-like* (74) and *AP1-like* (76) groups show major expansion in bamboo in terms of copy number, in comparison to rice (5, 5 and 4, respectively, **Figure 11**).

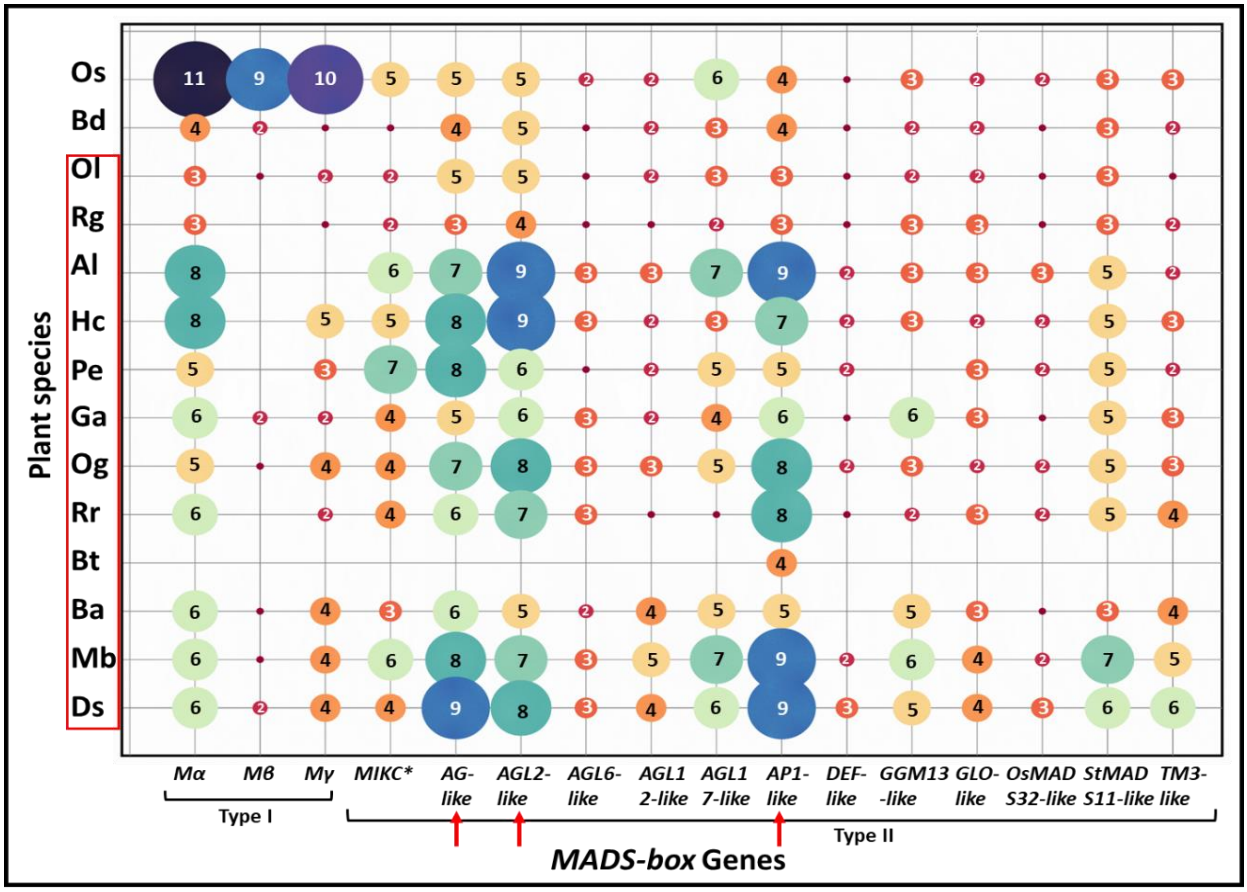

Figure 11. Bubble plot to demonstrate differential numbers of *MADS-box* genes identified in studied Poaceae members. The smallest red circle represents single copy. The copy numbers of genes mentioned within the bubble. The red rectangle represents all bamboos. The red arrow indicates the groups where the major gene expansions noted in bamboos.

4.7. Phylogenetic relationships of bamboo *MADS-box* gene homologs and *in silico* characterization of MADS-box proteins

As previously discussed, the *MADS-box* genes have been subdivided into 16 sub-groups, the phylogenetic relationship and the *in silico* characterization of the putative proteins have been studied for all the groups. Here, as a representative only the phylogeny and *in silico* characters of *AP1-like* genes have been shown.

AP1 is an important group of genes involved in flowering and studied in detail in various plant groups. These genes belong to the class A genes involved in sepal and petal development. In the tropical bamboo *B. tulda*, 4 copies of *AP1-like* genes have been isolated by targeted sequencing (Basak et al., 2025). This group involves 4 *MADS-box* genes in rice- *OsMADS14*, *OsMADS15*,

OsMADS18 and *OsMADS20*. This is the largest group containing 76 bamboo homologs (**Figure 12**). The phylogenetic tree shows 4 clusters corresponding to *MADS14*, *MADS15*, *MADS18* and *MADS20* groups. While *MADS14*, *MADS15* were grouped together, *MADS18*, *MADS20* were included in the other group. Such clear separation between *MADS14*, *MADS15* and *MADS18*, *MADS20* groups indicated their divergent evolutionary history.

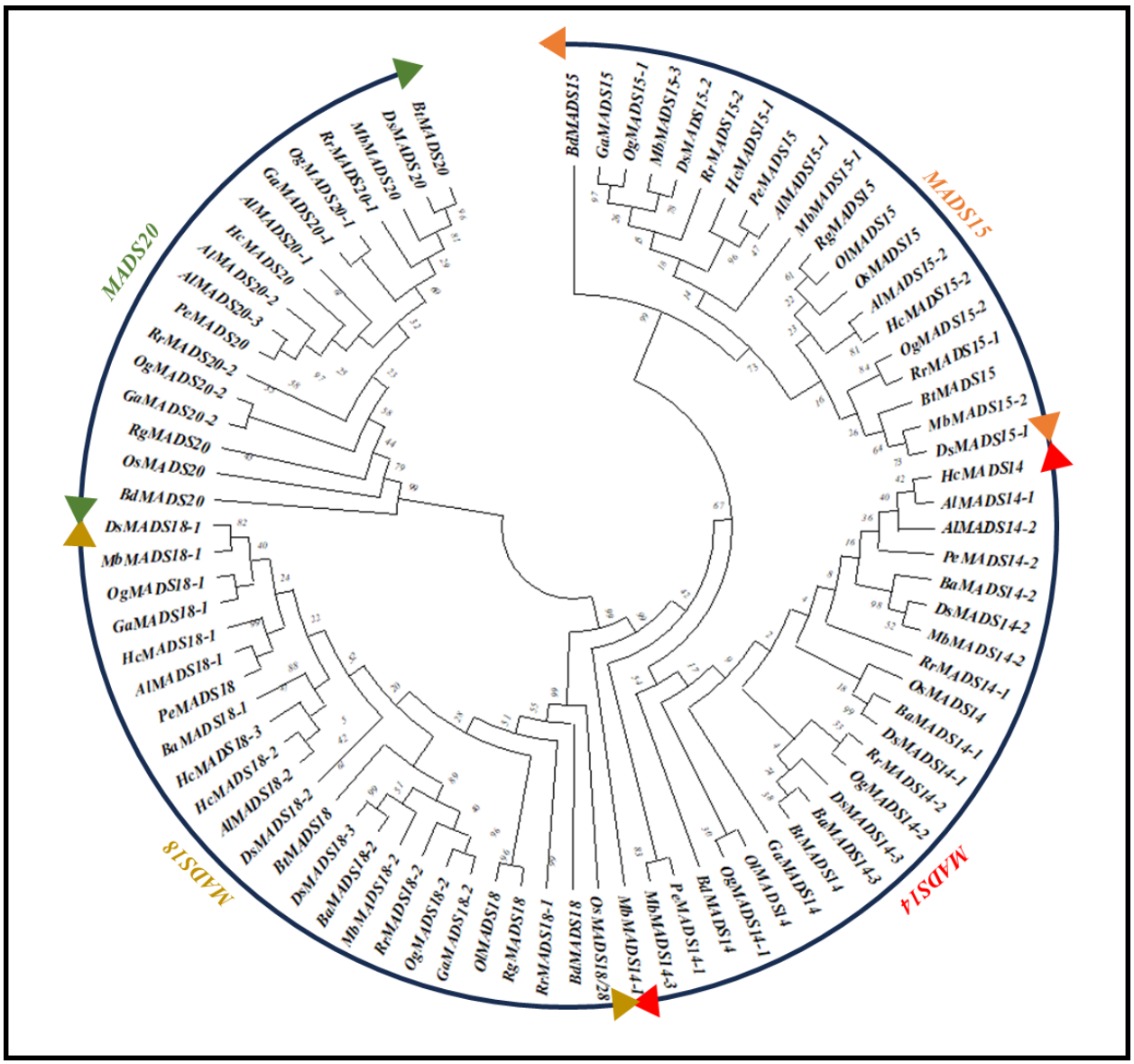

Figure 12. Phylogenetic comparison of *AP1/SQUA-like* genes identified in members of Poaceae.

The putative bamboo AP1-like proteins differ with respect to many characteristics. The protein length varied from 181 (AlMADS14-1) to 272 amino acids (RgMADS15, **Table 3**). All of them

are hydrophilic in nature based on their negative GRAVY value.

Table 3. *In silico* characteristics of bamboo AP1-like proteins. Abbreviations used- MW-molecular weight; kDa- kilo Dalton; pI- isoelectric point; PCRs- total number of positively charged residues (such as Arg + Lys); NCR- total number of negatively charged residues (such as Asp + Glu); II- instability index; AI-: aliphatic index; GRAVY-grand average of hydropathicity.

Plant name	Protein name	Length of amino acid	MW (KDa)	pI	NCRs	PCRs	II	AI	GRAVY
Olyra latifolia	OlMADS14	237	27410.49	9.20	31	39	55.69	83.46	-0.750
	OlMADS15	263	30177.41	9.10	31	38	65.08	78.33	-0.784
	OlMADS18	255	28843.78	9.17	31	37	54.63	81.06	-0.784
Raddia guianensis	RgMADS15	272	31307.67	9.03	32	38	62.27	81.10	-0.810
	RgMADS18	258	29047.67	8.72	32	35	59.01	77.87	-0.866
	RgMADS20	240	27230.97	6.20	36	33	59.66	86.21	-0.541
Ampelocalamus luodianensis	AlMADS14-1	181	20795.48	7.73	25	26	52.33	76.02	-0.871
	AlMADS14-2	244	28093.16	9.13	31	38	52.81	81.11	-0.730
	AlMADS15-1	259	29740.06	9.02	33	39	68.52	77.61	-0.778
	AlMADS15-2	257	29548.84	9.03	33	39	67.52	79.73	-0.786
	AlMADS18-1	257	29086.59	8.50	33	35	56.41	76.26	-0.907
	AlMADS18-2	257	28790.35	8.79	31	34	52.93	78.95	-0.821
	AlMADS20-1	241	27034.68	6.55	34	33	66.76	90.71	-0.549
	AlMADS20-2	248	27958.75	5.63	37	31	68.53	92.46	-0.478
	AlMADS20-3	246	27629.47	6.34	36	34	68.69	93.98	-0.489
Hsuehochloa calcarea	HcMADS14	183	21274.09	6.54	26	25	56.52	75.68	-0.907
	HcMADS15-1	259	29900.22	9.14	33	40	65.51	78.34	-0.805
	HcMADS15-2	257	29622.96	9.03	32	38	62.76	81.98	-0.723
	HcMADS18-1	257	29207.06	9.81	27	40	55.21	78.56	-0.882
	HcMADS18-2	257	28893.51	9.46	29	37	53.30	77.43	-0.881
	HcMADS18-3	253	28494.09	9.63	29	40	48.87	75.61	-0.894
	HcMADS20	239	27038.82	7.06	33	33	66.39	95.90	-0.485
Phyllostachys edulis	PeMADS14-1	214	24931.81	9.04	30	37	52.88	93.32	-0.648
	PeMADS14-2	245	28234.41	9.20	32	40	54.42	81.59	-0.738
	PeMADS15	259	29839.12	9.23	33	41	63.27	78.38	-0.795
	PeMADS18	257	28989.63	9.43	29	37	53.15	75.53	-0.878
	PeMADS20	240	27096.84	6.26	35	32	68.51	93.08	-0.482
Guadua angustifolia	GaMADS14	237	27304.26	9.35	32	41	62.25	83.12	-0.803
	GaMADS15	260	29892.17	9.37	31	40	62.38	81.04	-0.800
	GaMADS18-1	263	29727.52	9.28	32	38	52.45	76.05	-0.880

	GaMADS18-2	255	28843.73	9.08	31	36	54.03	85.25	-0.740
	GaMADS20-1	240	27332.90	5.62	41	34	57.03	95.13	-0.573
	GaMADS20-2	233	26518.14	6.92	33	33	67.66	87.51	-0.548
Otatea glauca	OgMADS14-1	233	27053.96	9.25	30	39	63.61	81.16	-0.778
	OgMADS14-2	244	28167.18	9.11	33	40	63.01	79.10	-0.818
	OgMADS15-1	258	29646.81	9.27	32	40	59.74	81.67	-0.797
	OgMADS15-2	260	30016.39	9.39	31	41	61.72	80.31	-0.784
	OgMADS18-1	257	28992.80	9.28	30	36	52.76	80.08	-0.784
	OgMADS18-2	255	28918.75	9.19	30	35	58.78	83.33	-0.793
	OgMADS20-1	239	27217.97	7.75	35	36	60.77	91.00	-0.657
	OgMADS20-2	240	27048.80	8.29	31	33	64.78	87.42	-0.561
Rhipidocladum racemiflorum	RrMADS14-1	246	28254.39	9.38	32	42	53.92	75.33	-0.807
	RrMADS14-2	244	28197.22	9.05	33	40	60.42	75.53	-0.856
	RrMADS15-1	260	30097.38	9.03	33	39	68.49	80.31	-0.800
	RrMADS15-2	260	29685.98	9.04	32	38	63.63	82.23	-0.716
	RrMADS18-1	224	25493.90	9.26	30	36	52.77	82.28	-0.852
	RrMADS18-2	255	28917.72	9.32	30	36	56.67	81.41	-0.835
	RrMADS20-1	240	27129.74	6.62	36	35	70.50	86.21	-0.633
	RrMADS20-2	247	28011.77	8.38	34	36	63.39	84.94	-0.665
Bambusa tulda	BtMADS14	244	28242.39	9.11	32	39	52.62	81.15	-0.763
	BtMADS15	261	30014.28	8.85	33	38	67.87	78.89	-0.761
	BtMADS18	230	25994.43	9.08	29	33	63.67	80.57	-0.782
	BtMADS20	240	27213.99	8.38	34	36	66.35	93.50	-0.579
Bonia amplexicaulis	BaMADS14-1	231	26823.04	9.75	26	43	50.26	76.41	-0.820
	BaMADS14-2	243	28019.02	8.72	34	38	54.15	77.86	-0.709
	BaMADS14-3	244	28106.22	9.11	33	40	60.19	81.15	-0.769
	BaMADS18-1	249	28221.80	9.32	32	38	59.82	81.49	-0.861
	BaMADS18-2	255	28958.81	9.18	31	36	59.32	81.41	-0.818
Melocanna baccifera	MbMADS14-1	188	22009.56	9.34	26	37	52.16	86.06	-0.760
	MbMADS14-2	242	27769.71	8.89	32	37	58.74	79.01	-0.719
	MbMADS14-3	233	26796.78	9.31	31	40	48.72	87.90	-0.670
	MbMADS15-1	260	30087.20	8.84	36	41	58.36	73.96	-0.847
	MbMADS15-2	259	29819.09	9.10	33	39	62.47	81.39	-0.758
	MbMADS15-3	248	28550.67	9.18	28	36	62.65	77.90	-0.806
	MbMADS18-	257	29017.66	9.28	32	38	58.83	78.95	-0.875

	1								
	MbMADS18-2	255	28942.76	9.04	32	36	52.37	82.59	-0.784
	MbMADS20	240	26914.61	7.03	32	32	67.58	93.92	-0.528
Dendrocalamus sinicus	DsMADS14-1	240	27717.94	9.56	28	41	58.26	75.96	-0.792
	DsMADS14-2	244	28060.17	9.13	32	39	54.08	79.55	-0.699
	DsMADS14-3	244	28134.36	9.35	31	41	58.84	83.11	-0.737
	DsMADS15-1	260	29808.08	8.75	34	38	64.00	82.96	-0.725
	DsMADS15-2	258	29348.53	9.10	29	36	62.20	80.19	-0.672
	DsMADS18-1	255	28709.45	9.43	31	39	60.11	79.57	-0.849
	DsMADS18-2	247	28154.87	9.58	30	39	58.18	81.70	-0.868
	DsMADS18-3	255	29018.90	9.18	31	36	57.54	82.94	-0.824
	DsMADS20	240	27098.86	7.06	35	35	65.66	93.50	-0.556

4.8. Syntenic relationship of *Phyllostachys edulis MADS-box* genes with rice and *Brachypodium*

The syntenic relationship was carried out to understand the possible consequence of gene duplication on the *MADS-box* genes of *P. edulis*. The syntenic relationship studies were analyzed for 6 selected groups of *MIKC*^{C} genes (*AG-like, AGL2-like, AGL17-like, AP1-like, GLO-like* and *StMADS11-like*) where the homologs of *P. edulis* is 3 or more. For each group of genes, the synteny was analysed against Rice and Brachypodium, which are 2 important members of Bambusoideae Oryzoideae Pooideae (BOP) clade and offers good quality genome to perform evolutionary analyses. Here, as a representative, syntenic relationship of *AP1-like* genes has been shown.

The *AP1-like* genes are distributed among 4, 12, 15, and 21 scaffolds of *P. edulis*. The 5 *AP1-like* genes of *P. edulis* are found to be syntenic with 4 *AP1-like* genes of *Brachypodium* (Chr1 and Chr4) and rice (Chr3, Chr7, and Chr12). One-to-one syntenic relationship is seen in case of *PeMADS18*: *BdMADS18* and *PeMADS20*: *BdMADS20* (**Figure 13**). Similar relationship could be obtained for *PeMADS18*: *OsMADS18* and *PeMADS20*: *OsMADS20*. In contrast, one-to-many syntenic relationship could be observed for *PeMADS14-1-PeMADS14-2*: *BdMADS14*, *PeMADS15*: *BdMADS14-BdMADS15* and *PeMADS14-1-PeMADS14-2-PeMADS15*: *OsMADS14-OsMADS15*.

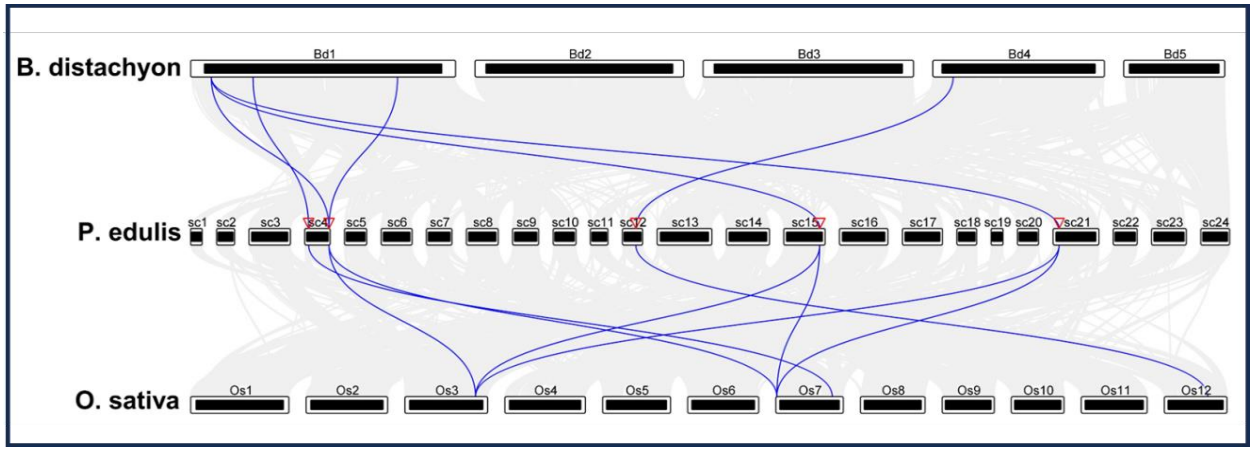

Figure 13. Synteny analysis of *AP1-like* genes among *Phyllosatchys edulis*, *Brachypodium distachyon* and *Oryza sativa*. The grey lines in the background indicate collinear blocks of these three genomes. The blue lines highlight the syntenic gene pairs among *P. edulis*, *B. distachyon* and *O. sativa*.

4.9. Expression analyses to study involvement of *MADS-box* genes in the reproductive development of bamboos

This study was based on transcriptome data which was available in 3 bio-replicates for various tissue stages for *P. edulis* only. The transcriptome data of tissue specific expression were obtained from 5 tissues. These were related to flowering (e.g., leaves from plant that flowered next year, leaves of flowering plants and flower florets) or from vegetative tissues (e.g., leaves from 3 weeks old seedling, leaves from 1 year old plant). Clustering of genes based on their expression pattern in these tissues indicate formation of 4 groups (**Figure 14**). The group 1 included 3 genes (*PeMADS18*, *PeMADS14-1* and *PeMADS65*), which were expressed in leaves of flowering plant and flower florets. Therefore, they are possibly involved in floral organ induction and differentiation. The group 2 included 9 genes (*PeMADS3-2*, *PeMADS68-2*, *PeMADS16-2*, *PeMADS26-2*, *PeMADS4-2*, *PeMADS32-2*, *PeMADS1*, *PeMADS34*, *PeMADS5-2*), those were expressed only in flower florets. They are possibly involved in floral organ differentiation. Group 3 included 7 genes (*PeMADS56*, *PeMADS18*, *PeMADS68-1*, *PeMADS60*, *PeMADS55*, *PeMADS62-2*, *PeMADS22-2*). These genes were primarily expressed in leaves of plants that flowered next year, which indicates their possible roles in flower induction. Three genes (*PeMADS47*, *PeMADS20*, *PeMADS65*) were included in group 4, which were expressed in leaves obtained from 3 weeks old seedlings and 1 year old plants, which might be related to vegetative

growth and development.

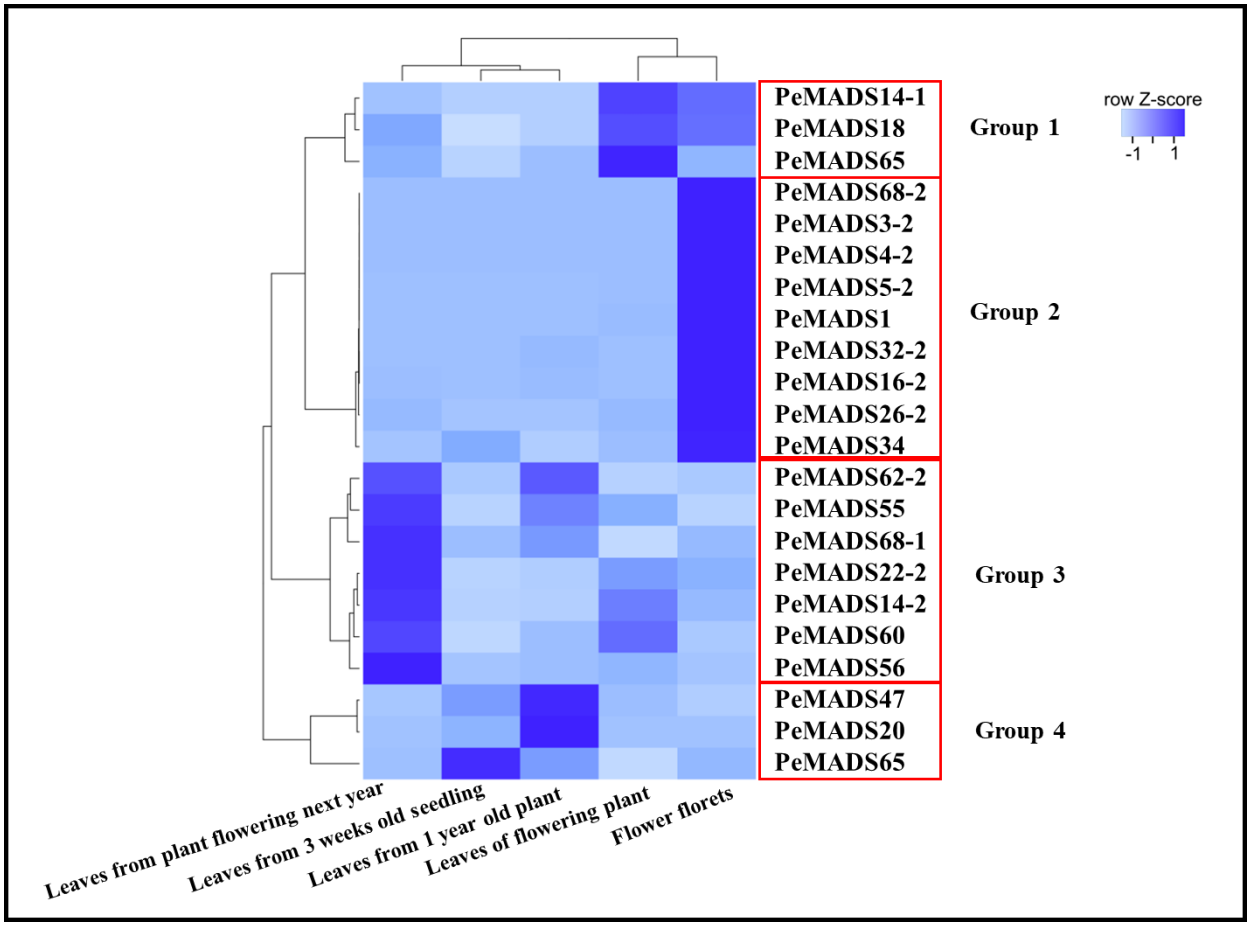

Figure 14. Heat map analysis of RNA-Seq transcriptome analysis for selected *MADS-box* genes from *Phyllostachys edulis*. The Heat map was generated using Heatmapper with average tpm values.

4.10. Floral organ specific and seasonal expression analysis of *Bambusa tulda AP1* genes

Four homologs of *AP1* genes have been identified from tropical bamboo *B. tulda* by targeted sequencing (*BtMADS14*, *BtMADS15*, *BtMADS18* and *BtMADS20*, Basak et al., 2025). The tissue specific expression data of all these homologs show the involvement of *BtMADS14* and *BtMADS15* in floral induction (Basak et al., 2025). The expression study of these genes in floral whorls such as glume (G), lemma+palea+lodicule (LPL), stamen (ST) and carpel (C) shows their highest expression in lemma, palea and lodicule (LPL), indicating their possible A gene function (**Figure 15A**). The expression levels of *BtMADS18* and *BtMADS20* were consistently very low

compared to *BtMADS14*, *BtMADS15*.

The seasonal expression pattern of *B. tulda AP1* genes were conducted in the leaves of flowering plants in summer, autumn, winter and spring. The expression of *BtMADS14*, *BtMADS15* and *BtMADS18* were upregulated in winter suggesting their possible involvement in the flower induction in the following spring. However, such trend could not be observed for *BtMADS20* (**Figure 15B**).

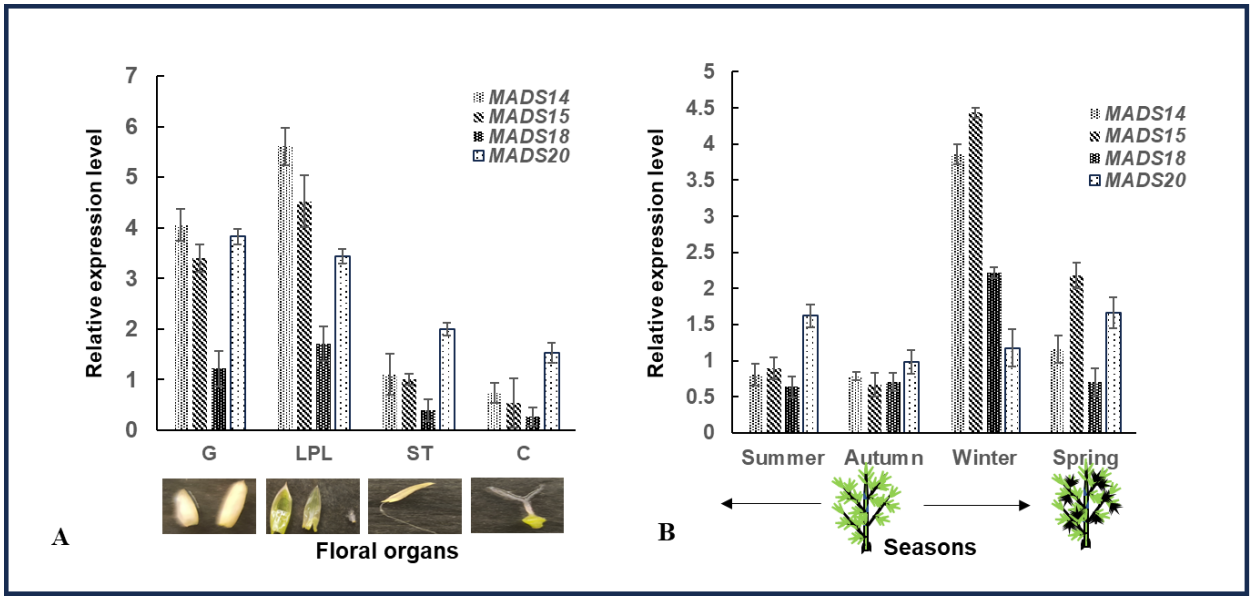

Figure 15. A. Study of expression levels of *AP1* genes in different floral organs of Bambusa tulda B. Study of expression levels of *AP1* genes in leaves of flowering plants in different seasons. Each bar represents the mean of three biological replicates ± standard error. Abbreviations used: G- Glume, LPL- Lemma+Palea+Lodicule, ST- Stamen, C- Carpel.

4.11. Conservation of M, I, K and C domains in *Bambusa tulda AP1* homologs

Being a member of *MADS-box* gene family, *AP1-like* genes contain four major domains, M (MADS), I (Intervening), K (Keratin-like) and C (C terminal). In all *AP1* homologs of *B. tulda*, the nuclear localization signal could be identified. The detailed *in silico* studies on the *B. tulda AP1* genes show conservation of M domain and important amino acids which are known to maintain its function (**Figure 16**). Complete I and K domains are also present. The C domain is also found to be present with conserved 6 amino acids long paleoAP1 motif.

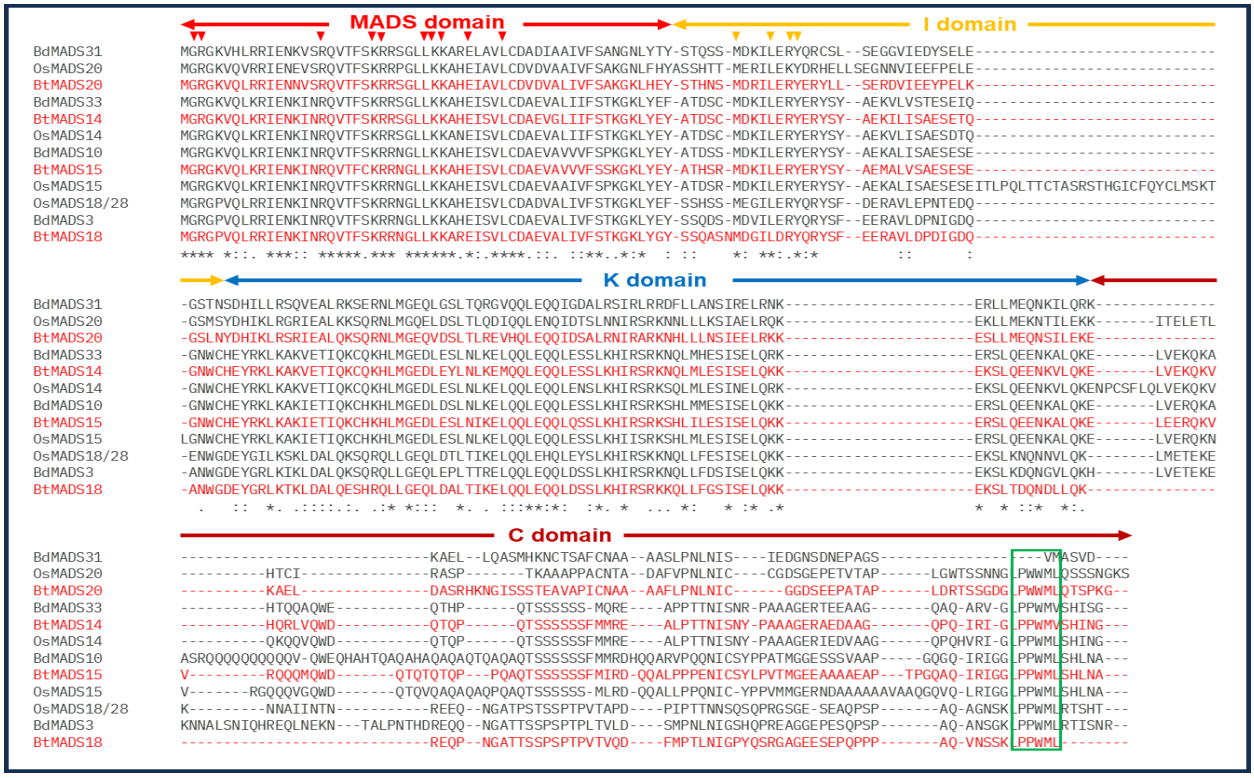

Figure 16. Multiple sequence alignment of amino acid sequences to identify MADS, I, K and C domains in rice, *Brachypodium* and *Bambusa tulda*. Functionally important amino acids in MADS and I domains are marked in red and yellow arrows, respectively, and the PaleoAP1 motif is marked in green box. Abbreviations used: Os- *Oryza sativa*, Bd- *Brachypodium distachyon*, Bt- *Bambusa tulda*.

❖ **Objective 3**

4.12. Identification, copy number comparison and phylogenetic relationship of *FT* gene homologs from bamboos

A total of 18 *FT* homologs has been identified from 12 Poaceae members by using 2 copies of rice FT proteins as queries. Among them, 17 homologs are from 11 sequenced bamboos. Among the bamboos, 6 bamboo species contained 2 copies each, while 5 bamboo species had 1 each and 1 copy is present in another (**Table 4**).

Table 4. List of *FT* gene homologs identified from sequenced bamboo species.

Gene	Plant name	Gene ID	Gene name	Identity (%)	E value	Length of amino acid	Database

Hd3a/ FT	*Oryza sativa*	LOC_Os06g06320.1	*OsHd3a/FT*	-	-	179	https://phytozome-next.jgi.doe.gov/
	Brachypodium distachyon	Bradi1g48830	*BdFT*	91	9e-121	177	https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v5_monocots/
	Olyra latifolia	Ol08g03590.mRNA1	*OlFT*	85	2e-114	177	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
	Raddia guianensis	RguEVM0014703.1	*RgFT*	86	2e-116	177	
	Ampelocalamus luodianensis	Alu06Cg16320.2	*AlFT1*	88	3e-117	177	https://bamboo.genobank.org/
		Alu06Dg02780.2	*AlFT2*	91	1e-121	178	
	Hsuehochloa calcarea	Hca06Cg14490.1	*HcFT1*	87	1e-114	177	
		Hca06Dg12920.2	*HcFT2*	89	9e-119	179	
	Phyllostachys edulis	PH02Gene17215.t1	*PeFT1*	89	1e-116	178	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		PH02Gene34011.t1	*PeFT2*	89	1e-116	178	
	Guadua angustifolia	GanEVM0044022.2	*GaFT*	86	4e-114	177	
	Otatea glauca	Ogl06Bg02840.1	*OgFT*	91	1e-121	177	https://bamboo.genobank.org/
	Rhipidocladum racemiflorum	Rhi06Bg02640.1	*RrFT*	89	1e-110	177	
	Bambusa tulda	KT003820	*BtFT1*	90	2e-119	178	https://www.ncbi.nlm.nih.gov/
		KT003821	*BtFT2*	90	2e-119	178	
		KU726232	*BtFT3*	88	4e-109	178	
		KX290774	*BtFT4*	88	4e-109	178	
	Bonia amplexicaulis	Bam06Ag10520.mRNA1	*BaFT1*	89	1e-117	177	https://gendbe.computational.bio.uni-giessen.de/cgi-bin/blast_interface.cgi
		Bam03Cg11900.mRNA1	*BaFT2*	88	2e-116	177	
	Melocanna baccifera	Mhu06Ag01900.1	*MbFT1*	90	2e-120	177	https://bamboo.genobank.org/
		Mhu03Cg04430.1	*MbFT2*	89	7e-115	177	
	Dendrocalamus sinicus	Dsi06Ag02090.1	*DsFT1*	89	3e-118	182	
		Dsi03Cg05490.1	*DsFT2*	88	1e-108	177	
RFT1	*Oryza sativa*	LOC_Os06g06300.1	*OsRFT1*	-	-	178	https://phytozome-next.jgi.doe.gov/

At least 1 homologous copy of *OsHd3a/FT* could be found in each of the studied bamboo species. In *B. tulda* 4 copies of *FT* have been identified, which is largest among all bamboos. However, homologous *OsRFT1* could not be found in any Poaceae members including bamboos (**Figure 17**).

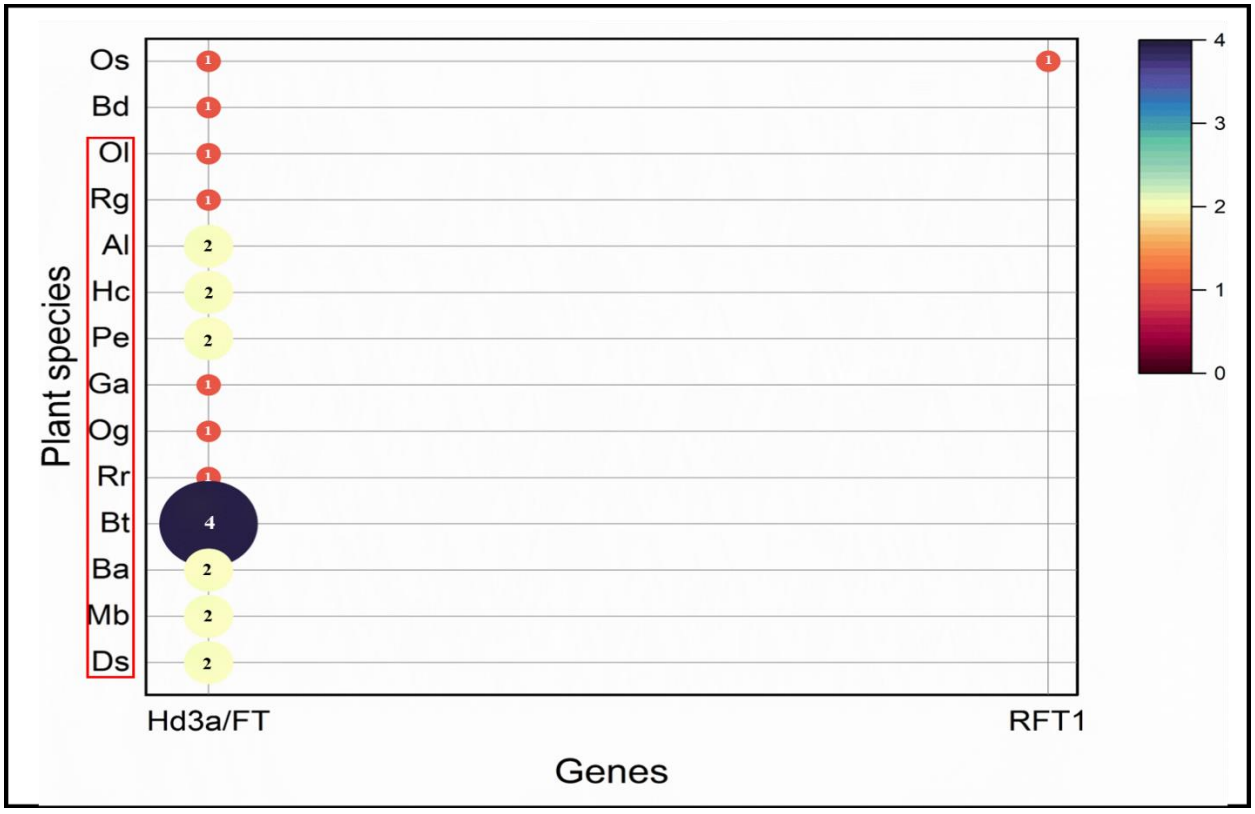

Figure 17. Bubble plot to demonstrate differential numbers of *FT* gene copies identified in studied Poaceae members. The copy numbers of genes mentioned within the bubble. The red rectangle represents all bamboos.

Phylogenetic analysis revealed clustering of all the *FT* homologs in *Hd3a/FT* clade, while *RFT1* was clearly separated out (**Figure 18**).

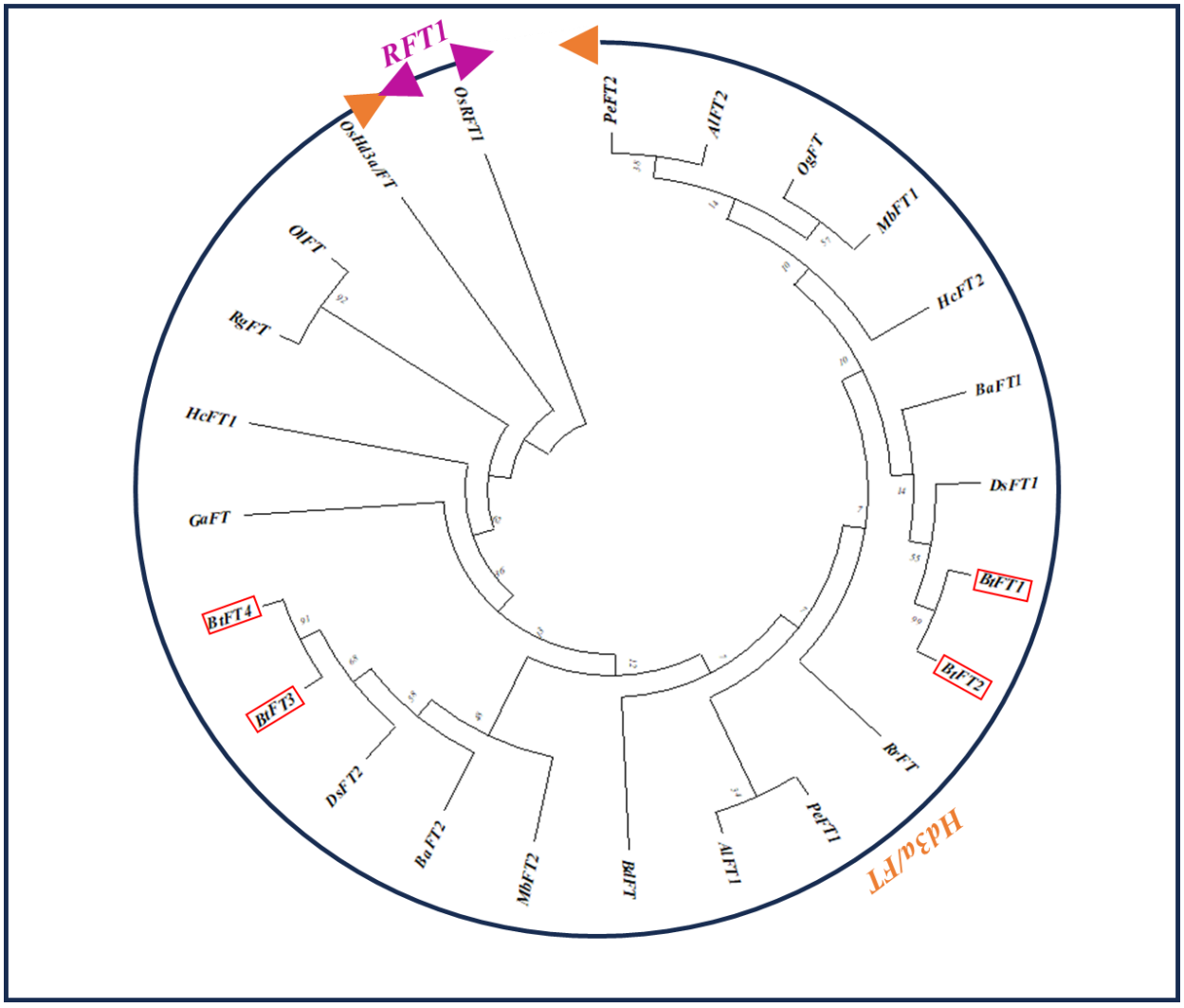

Figure 18. Phylogenetic tree of *FT* genes of studied Poaceae members. Genes identified by targeted sequencing in *B. tulda* have been highlighted in red boxes.

4.13. Conservation of domain and important amino acids in bamboo FT proteins and their *in silico* characterization

All bamboo FT proteins show conserved PEBP domain along with DPDxP, GxHR motifs (**Figure 19**). The L/IYN motif was exclusively found in FT proteins, but not in its close homolog FT-like proteins. This motif could also be detected in all bamboo Hd3a/FT members. The diagnostic PEBP motif of 116 amino acids was found in all putative bamboo FT proteins. The 5th amino acid of the PEBP domain, which is 64th position of the protein contains the Phe residue in case of Hd3a/FT, which is very important for binding to the 14-3-3 partner of floral activator complex. This residue

has been replaced by Ala in FT-like proteins. This amino acid is conserved among all FT proteins except MbFT2, BaFT2, DsFT2, BtFT3 and BtFT4, where Leu is present in this position. Similarly, the 101st position of FT protein contains an important Phe residue which is replaced by Ile in DsFT1, BtFT1, BtFT2, BaFT1 (**Figure 19**).

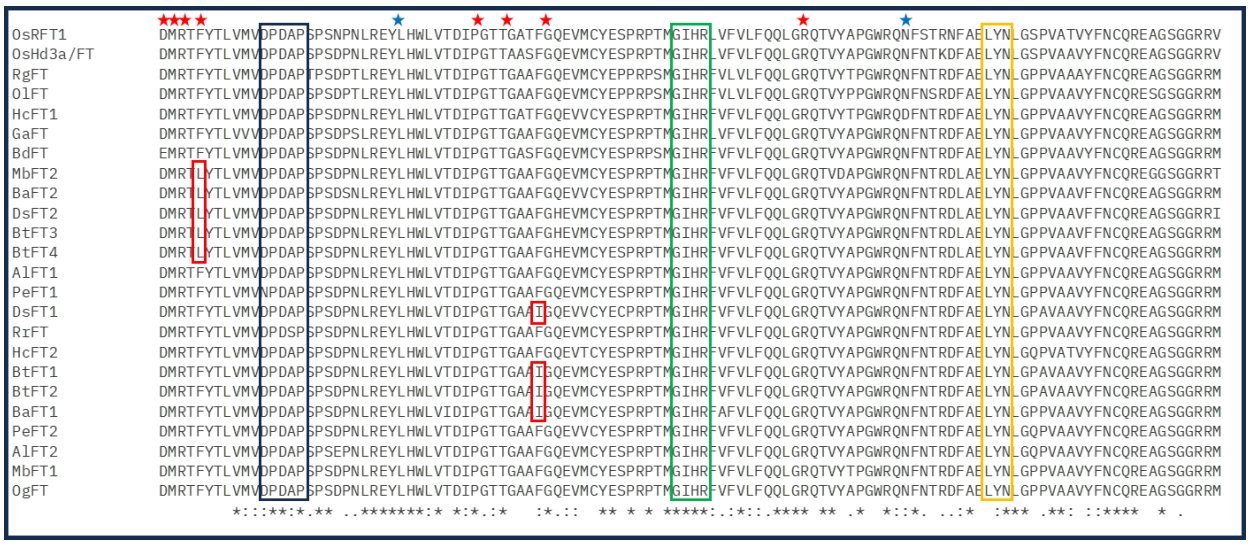

Figure 19. Multiple sequence alignment of amino acid sequences to identify important amino acids and motifs in PEBP domain of bamboos. The blue star indicates key amino acids determining *FT-like* and *TFL1-like* functions. The red star indicates amino acids interact with 14-3-3 protein. Black, green and yellow boxes represent conserved DPDxP, GxHR and L/IYN motifs, respectively. Red boxes represent deviation of conserved amino acids important for *FT* function.

The deduced protein sequence of bamboo FT varies from 177 (OlFT, RgFT, AlFT1, HcFT1, GaFT, OgFT, RrFT, BaFT1, BaFT2, MbFT1, MbFT2, DsFT2) to 182 amino acids (DsFT1). The predicted molecular weight is also highest for DsFT1 among all bamboo FT homologs. All the proteins show hydrophilic nature based on their negative GRAVY value (**Table 5**).

Table 5. Comparisons of *in silico* characteristics of bamboo FT proteins.

Plant name	Protein name	Length of amino acid	MW (KDa)	pI	NCRs	PCRs	II	AI	GRAVY
Olyra latifolia	OlFT	177	19866.67	8.54	17	19	31.01	72.54	-0.301
	OlFTL12	173	19385.17	8.54	16	18	39.15	71.45	-0.199
Raddia guianensis	RgFT	177	19801.55	7.75	17	18	22.72	70.90	-0.288
Ampelocalamus luodianensis	AlFT1	177	20096.91	8.52	17	19	43.54	69.27	-0.332
	AlFT2	178	20103.89	7.75	17	18	33.39	70.51	-0.322

	AlFTL12-1	163	18286.85	8.93	15	17	52.40	73.44	-0.269
	AlFTL12-2	173	19344.02	8.85	16	18	39.88	75.95	-0.220
Hsuehochloa calcarea	HcFT1	177	19870.57	6.10	19	18	33.72	70.34	-0.303
	HcFT2	179	20126.87	6.72	17	17	32.18	69.55	-0.297
	HcFTL12-1	173	19317.94	8.85	16	18	39.88	73.70	-0.247
	HcFTL12-2	169	18883.45	6.27	16	15	35.04	76.63	-0.210
Phyllostachys edulis	PeFT1	178	19973.78	8.52	16	18	30.90	71.57	-0.278
	PeFT2	178	20059.77	7.75	17	18	33.69	70.51	-0.337
	PeFTL12-1	173	19312.97	8.88	16	18	44.66	74.28	-0.224
	PeFTL12-2	173	19330.98	7.89	17	18	40.50	75.95	-0.221
Guadua angustifolia	GaFT	177	19716.47	8.52	16	18	36.68	74.75	-0.217
	GaFTL12	173	19358.01	7.90	17	18	42.36	73.70	-0.239
Otatea glauca	OgFT	177	19858.61	7.74	17	18	30.66	70.90	-0.308
	OgFTL12	173	19358.01	7.90	17	18	42.36	73.70	-0.239
Rhipidocladum racemiflorum	RrFT	177	19755.41	7.69	17	18	36.55	69.27	-0.314
	RrFTL12	173	19343.00	8.88	16	18	41.74	73.70	-0.239
Bambusa tulda	BtFT1	178	19871.60	6.82	17	17	28.34	72.70	-0.272
	BtFT2	178	19871.60	6.82	17	17	28.34	72.70	-0.272
	BtFT3	178	19902.64	6.41	18	17	25.11	72.70	-0.289
	BtFT4	178	19886.69	6.90	17	17	25.11	74.33	-0.246
	BtFTL12	173	19322.90	7.90	17	18	45.29	75.95	-0.277
Bonia amplexicaulis	BaFT1	177	19908.71	7.74	18	19	35.22	74.24	-0.306
	BaFT2	177	19702.39	7.82	17	18	31.99	75.88	-0.289
	BaFTL12-1	173	19289.89	7.89	16	17	43.88	73.70	-0.241
	BaFTL12-2	173	19330.94	8.88	16	18	46.49	72.02	-0.268
Melocanna baccifera	MbFT1	177	19922.65	7.74	17	18	25.81	68.14	-0.328
	MbFT2	177	19772.48	6.83	18	18	27.63	76.95	-0.290
	MbFTL12-1	173	19246.86	7.90	16	17	47.70	73.70	-0.202
	MbFTL12-2	173	19296.92	7.90	16	17	46.94	73.12	-0.236
	MbFTL12-3	173	19358.93	8.88	16	18	45.51	73.12	-0.282
Dendrocalamus sinicus	DsFT1	182	20244.09	7.69	17	18	30.98	74.34	-0.234
	DsFT2	177	19781.53	7.84	17	18	26.54	75.88	-0.267
	DsFTL12-1	173	19301.90	7.92	16	17	47.20	73.12	-0.218
	DsFTL12-2	173	19330.94	8.88	16	18	48.54	72.02	-0.268

4.14. Syntenic relationship among *FT* genes of *Phyllostachys edulis,* rice and *Brachypodium*

Rice and *Brachypodium* belongs to Bambusoideae Oryzoideae Pooideae (BOP) clade of family Poaceae. Both of them are phylogenetically close to bamboo and have genome with chromosome level assembly. Thus, the evolutionary analysis including synteny analysis could be performed using these genomes. The 2 copies of *FT* genes (*PeFT1* and *PeFT2*) were distributed on scaffolds 6 and 8 of *P. edulis* and show syntenic relationship with *OsFT* on Chr6 of rice and *BdFT* on Chr1 of *Brachypodium.*

Comparison of rice and *P. edulis FT* genes reveals many-to-one syntenic relationship for *PeFT1-PeFT2*: *OsRFT1* (**Figure 20**). Similarly, the synteny analysis between *P. edulis* and *Brachypodium* demonstrated many-to-one syntenic relationship among *PeFT1-PeFT2*: *BdFT.*

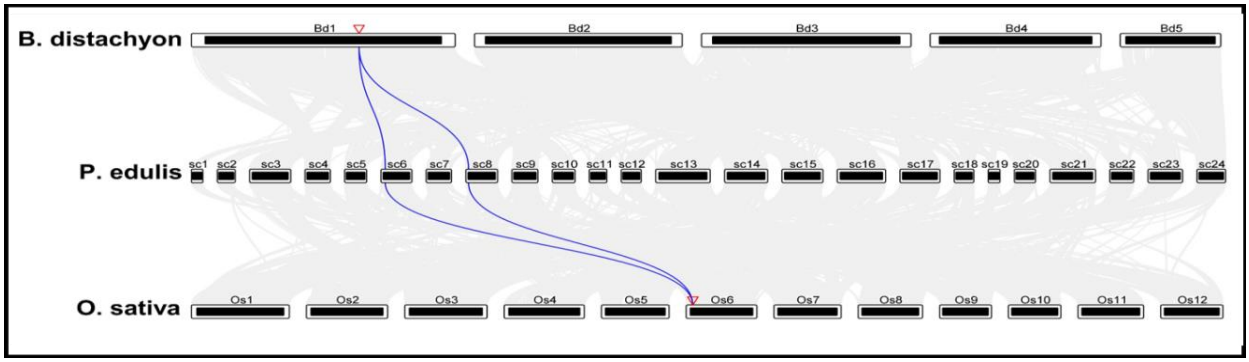
B. distachyon
Bd1
Bd2
Bd3
Bd4
Bd5
P. edulis
sc1 sc2 sc3 sc4 sc5 sc6 sc7 sc8 sc9 sc10 sc11 sc12 sc13 sc14 sc15 sc16 sc17 sc18 sc19 sc20 sc21 sc22 sc23 sc24
O. sativa
Os1 Os2 Os3 Os4 Os5 Os6 Os7 Os8 Os9 Os10 Os11 Os12

Figure 20. Synteny analysis of *FT* genes among *Phyllosatchys edulis*, *Brachypodium distachyon* and *Oryza sativa*. The grey lines in the background indicate collinear blocks of *P. edulis* with *B. distachyon* and *O. sativa* genomes. The blue lines highlight the syntenic *FT* gene pairs.

4.15. Analysis of phenotypic characters of *BtFT1* and *BtFT3* overexpressed Arabidopsis plants

The diurnal expression pattern of *BtFT1*, *BtFT2*, *BtFT3* and *BtFT4* suggested the possible involvement *BtFT3* and *BtFT4* in flowering, while *BtFT1* and *BtFT2* was not predicted to be involved (Dutta et al., 2018). Again, *in silico* characterization of BtFT proteins show amino acid changes between BtFT1/BtFT2 and BtFT3/BtFT4 clades (**Figure 19**). This is yet to be studied that if these amino acid changes have any effect on their differential expression. Therefore, *BtFT1* and *BtFT3* were selected to overexpress in Arabidopsis to investigate their roles in flower induction.

To ensure accurate selection of putative transgenic plants, seeds showing strong green fluorescence under fluorescent microscope were selected and grown for further analyses. Expression of the target genes were confirmed by the semi quantitative RT-PCR analyses in the transgenic plants by obtaining 100 bp long amplified band in the RT-PCR reaction.

The bolting time was recorded for 30 independent plants for each of the *p35S::BtFT1*, *p35S::BtFT3* and WT plants in LD condition. The bolting time of *p35S::BtFT1* and *p35S::BtFT3* plants were recorded at 32-38 (Mean: 35.63±0.37) and 30-38 (Mean: 35.17±0.47) days after germination (DAG) respectively. The WT plants took 40-45 (Mean: 42.30±0.33) DAG before the initiation of bolting (**Figure 21**).

Figure 21. Phenotypic comparisons of *p35S::BtFT1* (A) and *p35S::BtFT3* (B) Arabidopsis plants at 35 days after germination with WT plants. Plants were grown in LD (16-h light and 8-h dark). Bar = 5mm.

The rosette leaf numbers starting from 10 DAG until the initiation of bolting (i.e., 30 DAG) were counted for each of the transgenic plants compared to WT plants. For these two transgenic plants, no significant alteration with respect to the rosette leaf numbers (12.67±0.31 for *p35S::BtFT1* and 12.53±0.29 for *p35S::BtFT3*) were noticed compared to WT (11.80±0.36, **Figure 22A**). The statistical significance was measured via one-way ANOVA test.

The average number of seeds per individual silique and the silique length were also calculated for 30 individuals for each of the transgenic and WT plants. The average seed number was 44.30±0.61 for WT, 44.00±0.79 for *p35S::BtFT1* and 44.43±0.75 for *p35S::BtFT3* (**Figure 22B**). The average silique length and morphology (**Figure 22C**) were found to show no significant alteration compared to WT.

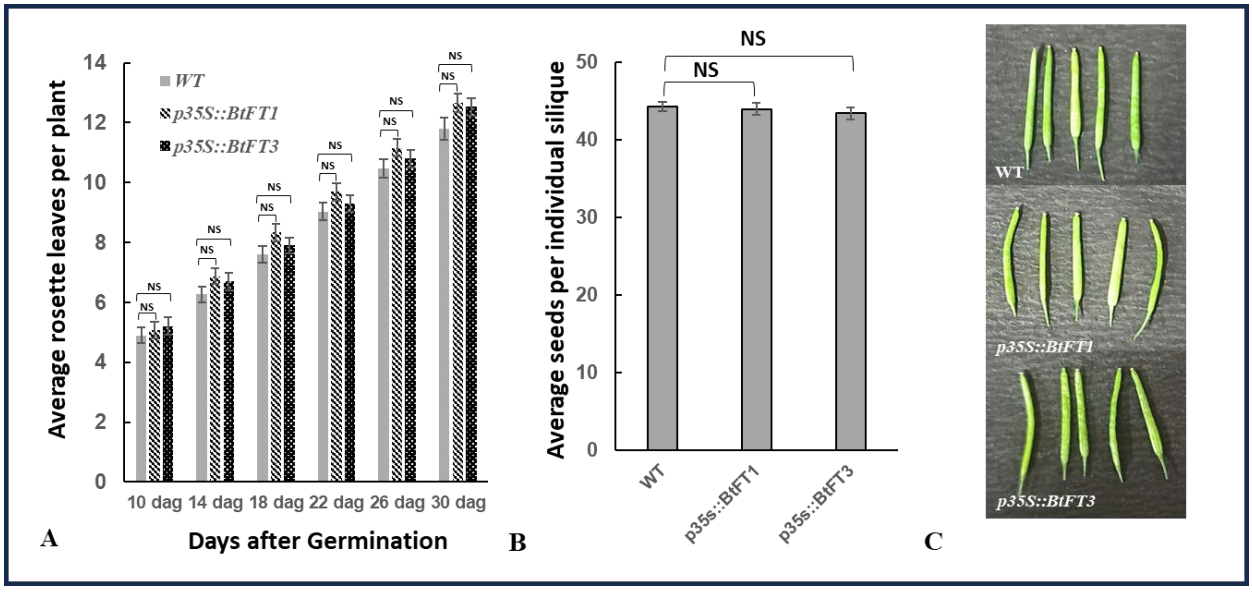

Figure 22. A. Comparison of rosette leaf numbers, B. average number of seeds per individual silique, C. morphology of siliques obtained from WT and transgenic *p35S::BtFT1* and *p35S::BtFT3* Arabidopsis plants. Rosette leaf numbers were counted starting at 10 days after germination (DAG) until 30 DAG. Plants were grown in LD (16-h light and 8-h dark). The statistical significance was evaluated at p-value ≤0.05 using a one-way ANOVA and two-sample t-test for comparing rosette leaf numbers (n=30 plants) and seeds per individual silique (n=30 siliques), respectively. Each value is displayed as a mean ± standard error. "ns"- not statistically significant.

4.16. Expression analysis of selected downstream genes in *BtFT1* and *BtFT3* overexpressed plants

The RT-qPCR analysis showed higher expression of approximately 12 folds of *BtFT1* (**Figure 23A**) and 48 folds of *BtFT3* (**Figure 23B**) in the respective transgenic plants and the fold changes in expression were statistically significant at p≤0.01 in comparison to WT plants.

It is already known that, FT forms a protein complex with FD which ultimately activates the flower inducer genes, such as *AP1*. In the *p35S::BtFT1* and *p35S::BtFT3* Arabidopsis plants, the elevated expression of the *AtAP1* (**Figure 23C**) indicated these gene's involvement in accelerated flowering in the transgenic lines. All the mentioned gene expressions were found to differ significantly in transgenic plants compared to WT in a two-sample t-test (at p-value ≤0.05).

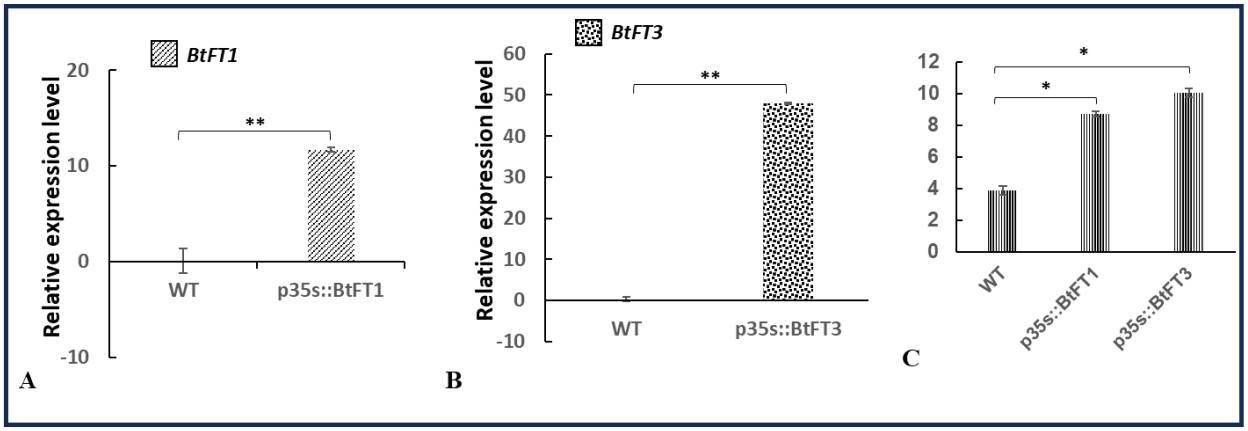

Figure 23. A. Transcript levels of *BtFT1* in WT and *p35S::BtFT1* plants B. Transcript levels of *BtFT3* in WT and *p35S::BtFT3* plants C. *AtAP1* expression study in WT, *p35S::BtFT1, p35S::BtFT3* plants. Each bar represents the mean of three biological replicates ± standard error. Abbreviations used: WT- Wild type, *AtAP1*- *A. thaliana APETALA1*. "*"- statistical significance at p-value ≤0.05, ""- statistical significance at p-value ≤0.01 in two sample t-test.**

4.17. Analysis of phenotypic characters of *BtMADS14* overexpressed Arabidopsis plants

From the tissue specific and seasonal expression analysis, *BtMADS14* and *BtMADS15* are found to be involved in flower induction and they are somewhat functionally redundant. Therefore, *BtMADS14* were selected as a representative *AP1* gene to ectopically express on *A. thaliana* Col-0 plants.

To ensure accurate selection of target gene inserted transgenic plants, seeds showing strong green fluorescence under fluorescent microscope were selected and grown (**Figure 24A**). The expressions of the *BtMADS14* were confirmed by the semi quantitative RT-PCR analyses in the transgenic plants (**Figure 24B**).

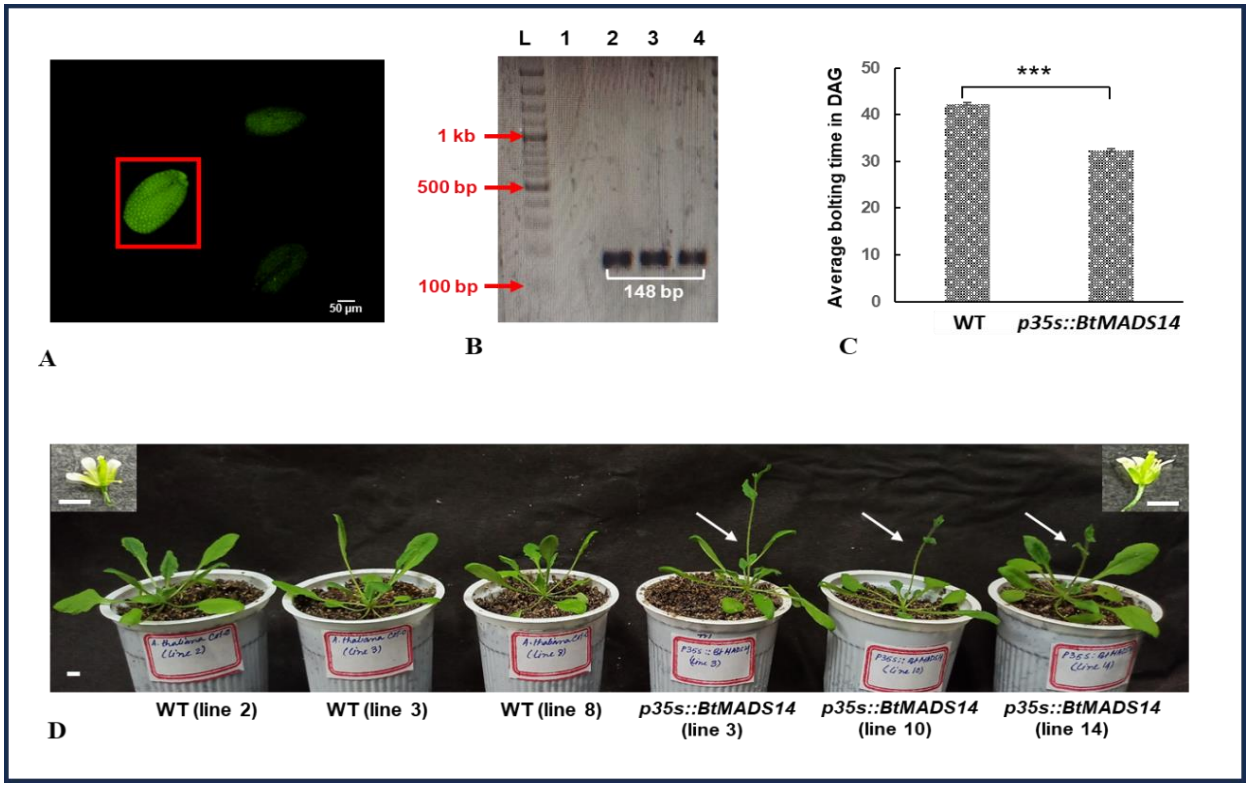

Figure 24. A. Confirmation of insertion of target genes by observing green fluorescence in putatively transgenic Arabidopsis seeds (red box) B. Confirmation of expression of *BtMADS14.* Lane 1: wild type (WT) Arabidopsis plant and lanes 2-4: *p35S::BtMADS14* Arabidopsis plants C. Comparison of average bolting day between WT and transgenic *p35S::BtMADS14* Arabidopsis plants grown in LD (16-h light and 8-h dark). The statistical significance was evaluated using a two-sample t-test (n=30 plants). Each value is displayed as a mean±standard error D. Phenotypic comparisons of WT and *p35S::BtMADS14* Arabidopsis plants at 35 days after germination. The inflorescence stalks are marked in white arrow. An individual flower of WT (left hand inset) and *p35S::BtMADS14* (right hand inset) has been presented. Abbreviations used: L- Molecular ladder, Bar = 5mm., "*"- statistical significance at p-value <0.001.**

The bolting time was recorded for 30 plant lines for each of the *p35S::BtMADS14*, and WT plants in LD condition. The bolting of *p35S::BtMADS14* and WT plants were recorded at 30-37 (Mean: 32.40±0.36) and 40-45 (Mean: 42.30±0.33) days after germination (DAG), respectively (**Figure 24C, D**).

The rosette leaf numbers starting from 10 DAG to the initiation of bolting (i.e., 30 DAG) were

also calculated for *p35S::BtMADS14* and WT plants. The rosette leaf numbers altered significantly (13.43±0.44) compared to WT (11.80±0.36) at 30 DAG (**Figure 25A**). The statistical significance was measured via one-way ANOVA test.

The average number of seeds per individual silique and the silique length were calculated for 30 individuals for each of the transgenic and WT plants. The average seed number was 44.30±0.61 for WT, 44.63±0.66 for *p35S::BtMADS14* plants (**Figure 25B**). The average silique length was found to be 1.56±0.04 cm and 1.60±0.03 cm for the WT and *p35S::BtMADS14* plants, respectively (**Figure 25C**). The two-sample t-test showed no significant alteration in both the seeds per silique and the silique length compared to WT.

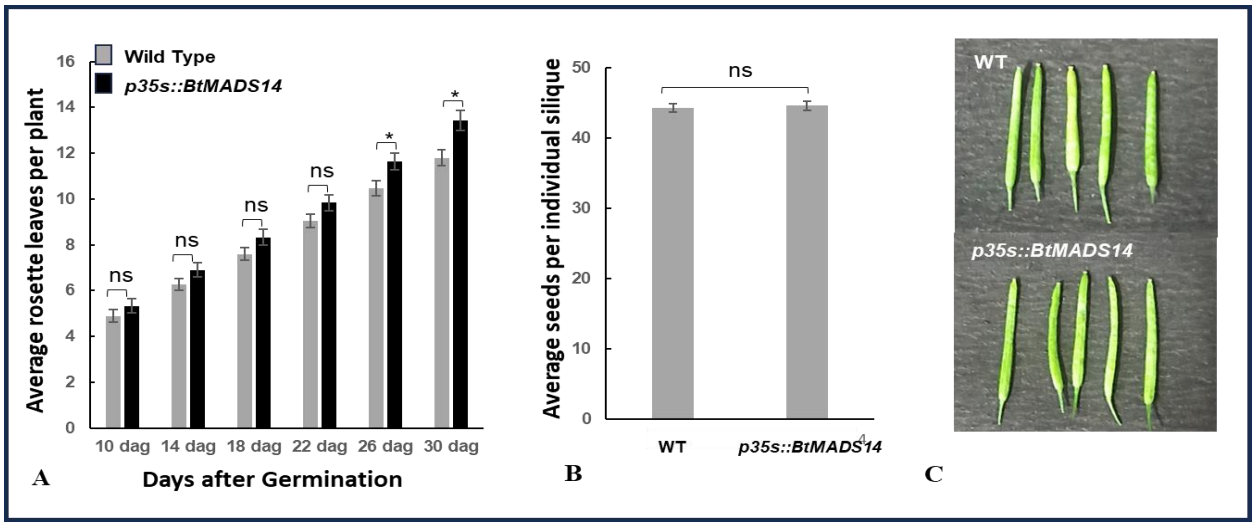

Figure 25. A. Comparison of rosette leaf numbers per plant B. average number of seeds per individual silique C. morphology of siliques of WT and *p35S::BtMADS14* Arabidopsis plants. Rosette leaf numbers were counted starting at 10 days after germination until day 30. Plants were grown in LD (16-h light and 8-h dark). The statistical significance was evaluated at p-value ≤0.05 using a one-way ANOVA and two-sample t-test for comparing rosette leaf numbers (n=30 plants) and seeds per individual silique (n=30 siliques), respectively. Each value is displayed as a mean±standard error. "ns"- not statistically significant, "*"- statistical significance at p-value <0.05.

4.18. *In silico* prediction of interacting partners for BtMADS14 protein

To understand the possible flower inductive regulatory pathway and targets of BtMADS14, the protein-protein interaction study was necessary. The interaction network of BtMADS14 could not

be predicted due to absence of transcriptome data. Thus, the possible protein-protein interaction network was predicted based on the homologous *P. edulis* proteins. PeMADS14-2 was used as the homolog of BtMADS14 in *P. edulis*.

The protein-protein interaction network of PeMADS14-2 shows 24 interacting protein partners, among which 8 are MADS-box proteins (2 copies for each of PISTILLATA, AGAMOUS-LIKE 20 and one copy for each of SEPALLATA 1, AGAMOUS, SHORT VEGETATIVE PHASE, FRUITFULL, **Figure 26**). Further, RT-qPCR validation of *FRUITFULL* gene was carried out in *p35s::BtMADS14* Arabidopsis plants.

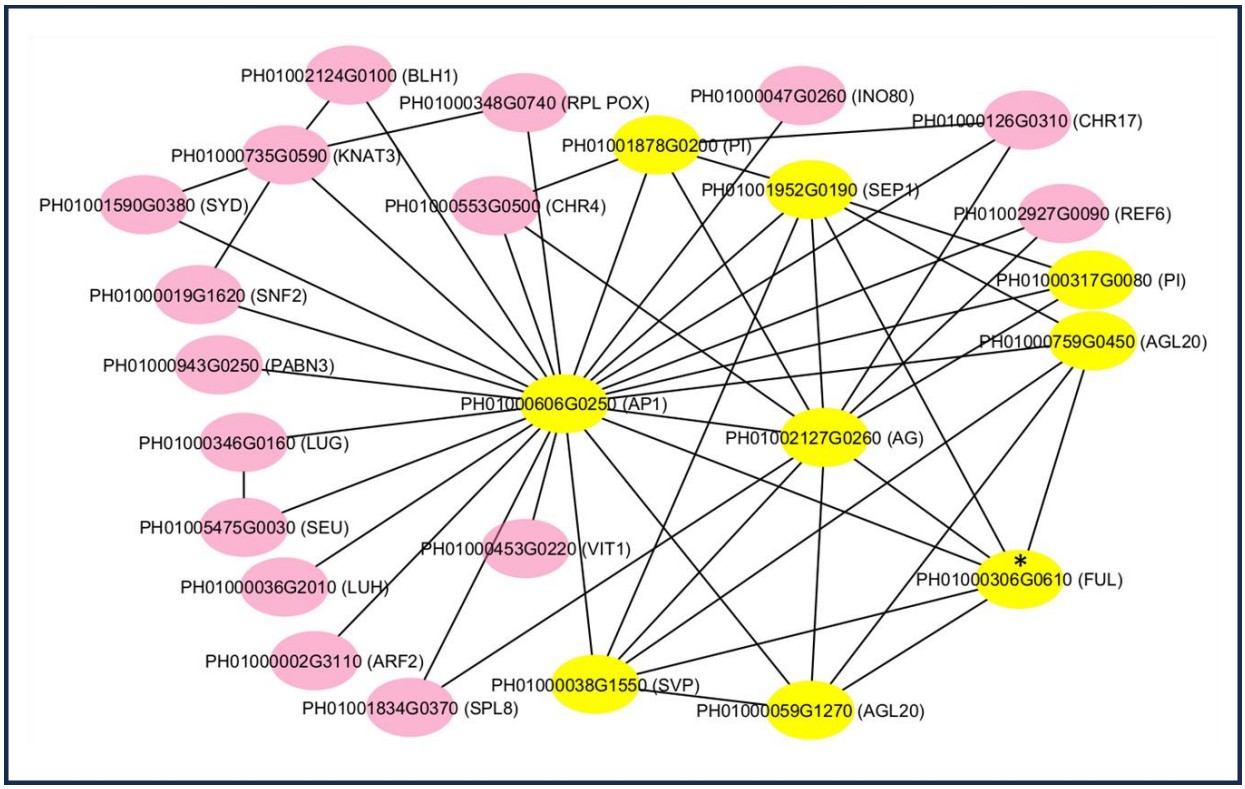

Figure 26. Protein-protein interaction network of PeMADS14-2 (AP1) protein. The proteins highlighted in yellow are MADS-box proteins and the candidate protein used for further experimental validation by RT-qPCR was marked by "*".

4.19. Expression analysis of selected downstream genes in *BtMADS14* overexpressed plants

The RT-qPCR analysis showed ~43 folds high expression of *BtMADS14* (**Figure 27A**) in the transgenic plants compared to WT plants. The *AtAP1* and *AtFUL* was selected as downstream target of BtMADS14 based on previous studies as well as protein-protein interaction study. In the

p35S::BtMADS14 plants elevated expression of both *AtAP1* and *AtFUL* were noticed (**Figure 27B**) indicating their possible involvement in accelerated flowering. The mentioned gene expressions were found to differ significantly in transgenic plants compared to WT at p-value <0.05 in a two-sample t-test.

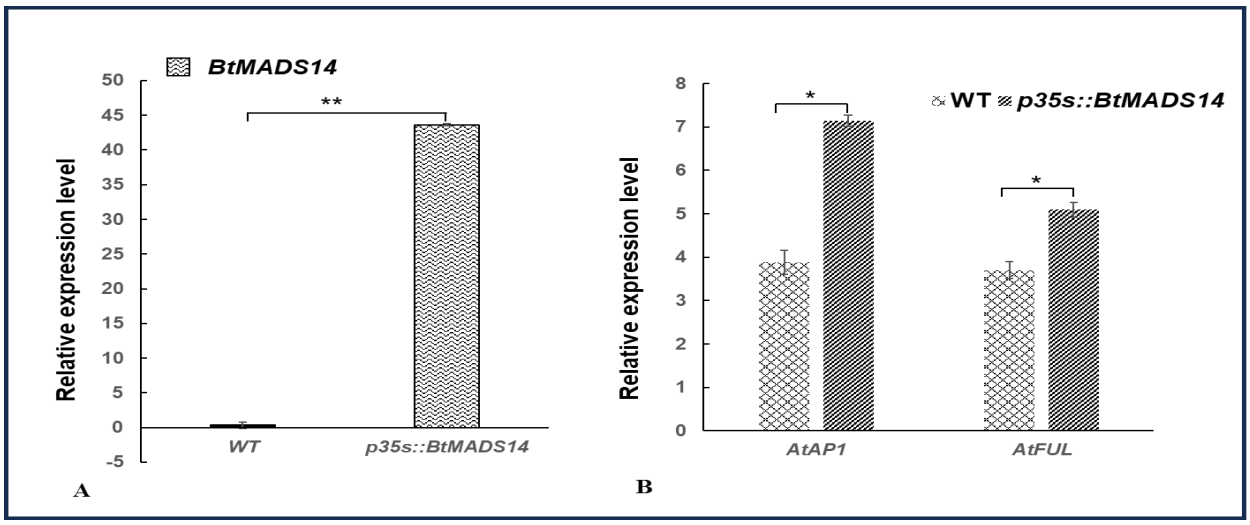

Figure 27. A. Expression levels of *BtMADS14* in WT and *p35S::BtMADS14* plants B. *AtAP1* and *AtFUL* expression study in WT and *p35S::BtMADS14* Arabidopsis plants. Each bar represents the mean of three biological replicates ± standard error. Abbreviations used: WT- Wild type, *AtAP1- A. thaliana APETALA1, AtFUL- A. thaliana FRUITFULL*. "*"- statistical significance at p-value ≤0.05, ""- statistical significance at p-value ≤0.01 in two sample t-test.**

5. Discussion

❖ **Objective 1**

5.1. *Bambusa tulda VIL* and their transcriptional target genes indicate their responsiveness towards vernalization and diurnal variation

Expression analyses of *VIL* genes on exposure to cold have most extensively conducted in Arabidopsis (Kim and Sung, 2013; Pyo et al., 2014). The Arabidopsis *VIL1* is constitutively expressed throughout the year, but its enhanced expression was detected during exposure to cold (Pyo et al., 2014). The *VIL2* transcript level was enhanced during early exposure to cold, in contrast to *VIL3* which was elevated at the late exposure stage (Kim and Sung, 2013). Similarly, the

expression pattern of *B. tulda VIL1* and *VIL2* gene showed clear upregulation during winter (**Figure 9A, B**). However, unlike Arabidopsis, increase in the expression of *BtVIL3* was noted during winter. Such differential response to cold exposure in terms of expression level have previously been recorded in *Aegilops tauschii*. Among 3 copies of *VILs*, only *AetVIL2* level was found elevated after one week of exposure to cold suggesting its clear involvement in the vernalization responsive pathway (Koyama et al., 2012).

Similar to rice, the diurnal expression pattern of *BtVILs* showed higher expression level in SD than LD. Particularly, expression levels of *BtVIL2* and *BtVIL3* were higher and was maximum at 8pm (**Figure 10**). In rice, two major expression peaks for *OsVIL2* (*~LC2*) were detected at 6pm and 11 pm in SD (Wang et al., 2013). However, for *OsVIL3* (*~OsVIL2*) the two major peaks were detected at later time points, which were at 1am and 3 am in SD (Wang et al., 2013).

OsVIL1 and *OsVIL2* in SD, promotes the rice flowering via the epigenetic silencing of the target gene *OsLF* (Wang et al., 2013). *OsVIL1* represses flowering by inducing *Ghd7* (Yoon et al., 2021). Again, *OsVIL2* represses *OsLFL1* (*~OsLF*) via epigenetic regulation and thereby inducing flowering (Yang et al., 2013). *OsLFL1* attenuates the expression of *Ehd1* via binding to its promoter and represses flowering (Peng et al., 2007). Similarly, in *B. tulda*, the expression pattern of *Ehd1* was opposite to *LFL1* (**Figure 10E, G**). Similar trend was also observed for *BtVIL* and *BtLFL1* genes.

❖ **Objective 2**

5.2. Clear shift in gene expression pattern between *BtMADS14, BtMADS15* vs. *BtMADS18, BtMADS20* like other Pooideae members

It is known that *AP1-like MADS-box* genes are involved in various developmental processes in plants and thus their tissue-specific expression varies across grasses. In rice, *OsMADS14*, *OsMADS15* and *OsMADS18* are expressed in broader range of tissues including vegetative tissues, inflorescence meristem, fertile bracts, stamen and carpel (Wu et al., 2017). However, the expression of *OsMADS20* was restricted only to the vegetative tissues. Much higher expression level of *OsMADS14* could be observed compared to *OsMADS15*, *OsMADS18* and *OsMADS20*. The *OsMADS15* was highly expressed in inflorescence meristem and fertile bract, while

OsMADS18 was more abundant in vegetative tissues (Wu et al., 2017).

In *Brachypodium*, transcript level of *BdMADS33* (*~BdMADS14/OsMADS14*), *BdMADS10* (*~BdMADS15/OsMADS15*), *BdMADS3* (*~BdMADS18/OsMADS18*) were high in fertile bracts (lemma, palea), and stamen (Wei et al., 2014). The *BdMADS3* was also primarily expressed in young seeds. The transcript of *BdMADS31* (*~BdMADS20/OsMADS20*) was detected only in leaf. Among 3 *AP1* genes in wheat, *WFUL1/VRN1* (*~OsMADS14*) and *WFUL3* (*~OsMADS18*) were detected in vegetative bracts, young spikes, anthers and carpels. The transcript level of *WFUL2* (*~OsMADS15*) was found in lemma and palea only (Kinjo et al., 2012).

A comparative study on *AP1* gene expression in bamboos showed the elevated expression of *MADS14* and *MADS15* in *B. tulda* (**Figure 15**), *MADS2* in *P. praecox* (Lin et al., 2009) and *MADS14* in *P. edulis* (Cheng et al., 2017) in the early stage of floral development indicating their possible role in floral induction. The transcript accumulation of *P. edulis MADS15* during the transition from SAM to IM and early flowering phenotype of *P. edulis MADS15* overexpressing Arabidopsis plants collectively indicate its possible role in flower induction (Cheng et al., 2017). In *B. tulda*, the expression level of *BtMADS18* and *BtMADS20* were lower than the other 2 members of the *AP1* group. This result is consistent with other findings on *P. praecox MADS1* (Lin et al., 2009), *P. edulis MADS18-1* and *MADS18-2* (Cheng et al., 2017). Similar to other Pooids, the tissue specific expression pattern of *BtMADS18* was clearly distinct from *BtMADS14* and *BtMADS15* (Basak et al., 2025). Collectively, the *AP1* homologs in *B. tulda* displayed A gene function (**Figure 15**), which is constant to its known function in other grasses.

❖ **Objective 3**

5.3. Copy number expansion, but no major alteration in phylogenetic pattern of bamboo *FT* and *AP1* gene homologs

FT-like genes are member of PEBP family genes which are involved in the regulation of floral transition in angiosperms. The molecular evolution studies suggested that *FT-like* genes originated during the evolution of the flowering plants (Pin and Nilsson, 2012), i.e., in non-flowering plants, there is no evidence for the presence of *FT*. *AP1* is a MADS-box family of transcription factors which play vital roles in the development of floral meristem and organs (Alvarez-Buylla et al.,

2006).

Till date, 13 *FT*-like and 4 *AP1* homologs have been identified in rice (Arora et al., 2007; Halliwell et al., 2016). Among 13 *FT*-like genes, only 3 are involved in flowering (Gu et al., 2022), while all 4 *AP1* homologs were found to be involved in flowering (Fornara et al., 2004; Wu et al., 2017). In *Brachypodium*, 2 *FT* gene homologs and 4 *AP1* genes were identified (Wei et al., 2014; Qin et al., 2017).

Majority of the bamboo genomes underwent extensive polyploidization events, which leads to existence of additional gene copies in bamboo genomes. Previously, in hexaploid bamboo *B. tulda*, 4 copies of *FT* genes (*BtFT1*, *BtFT2*, *BtFT3* and *BtFT4*) and in tetraploid bamboo *P. edulis*, duplicated copies of *AP1* genes such as *MADS15* (*PeMADS28*, *PeMADS41*) and *MADS18* (*PeMADS2*, *PeMADS13*) have been reported (Dutta et al., 2018; Zhang et al., 2018). In this study, higher *MADS14* were found in hexaploid *B. amplexicaulis* and tetraploid *P. edulis*, more than a copy of *MADS15* and *MADS18* were detected in *B. amplexicaulis* and *G. angustifolia* (**Figure 11**). Similarly, higher copies of *Hd3a/FT* were detected for most of the tetraploid (*H. calcareas*, *A. luodianensis*, *P. edulis*) and hexaploid (*B. amplexicaulis*, *M. baccifera*, *D. sinicus*) bamboo species, while *RFT1* could not be detected (**Figure 17**). This result suggested the retention of duplicated gene copies in highly polyploid bamboo genome (Guo et al., 2019). Previously similar expansion of gene copy numbers was detected in tetraploid *P. edulis* and hexaploid *B. tulda*. A total of 24 and 41 gene copies of *KNOTTED1-like homeobox* (*KNOX*) and *High-affinity K+/K+ uptake proteins/K+ transporter* (*HAK/KUP/KT*), respectively were detected in *P. edulis* which was 13 and 27 copies, respectively in rice (Jiao et al., 2024; Guo et al., 2024). Similarly, higher copy number of developmental genes such as *CONSTANS* and *FD* have been reported in *B. tulda* (Dutta et al., 2018; Dutta et al., 2021) compared to rice.

Phylogenetic analysis revealed the appearance of 4 distinct phylogenetic groups for bamboo *AP1* homologs (**Figure 12**), while FT homologs form a single cluster (**Figure 18**). *OsRFT1* forms an outgroup and lies next to *OsHd3a*. Previous report also suggested that *RFT1* is the closest homologue of *Hd3a* than that of the *FTLs* and along the same line, transgenic rice plants overexpressing *RFT1* flowered early, like that of the *Hd3a*-overexpressing plants (Izawa et al., 2002; Kojima et al.,2002).

The phylogenetic closeness of *MADS14* and *MADS15* revealed their possible common ancestry. The high sequence similarity of bamboo *MADS14* and *MADS15* indicated their redundant

function. It is reported that *MADS14* and *MADS15* were originated after two rounds of duplication in rice and other Pooid grasses (Vandepoele et al., 2003; Paterson et al., 2004; Yu et al., 2005, Preston and Kellogg, 2006). *Brachypodium FUL1* (~*OsMADS14*) and *FUL2* (~*OsMADS15*) were originated via a duplication event occurring at the base of Poaceae, while *FUL3* (~*OsMADS18*) was invented by a duplication event at the base of monocots (Li et al., 2016).

5.4. Ectopic expression of *BtMADS14, BtFT1* and *BtFT3* caused early flowering in Arabidopsis

Ectopic expression of *BtMADS14* caused early flowering in transgenic Arabidopsis (**Figure 24D**), but no morphological alteration of the flower morphology could be observed suggesting the gene's role in flower induction.

In the *p35S::BtMADS14* plants, the bolting time is reduced. Similar result was obtained when other Bambusoideae *AP1* genes were ectopically expressed (Lin et al., 2009; Cheng et al., 2017). Overexpression of *OsMADS14* in rice also causing early flowering suggested its role in inflorescence meristem determination (Jeon et al., 2000). *OsMADS15* is sequentially close paralog of *OsMADS14* and hence show functional redundancy to some extent (Kyozuka et al., 2000). *P. edulis PheMADS15* overexpressing Arabidopsis resulted in early flowering (Cheng et al., 2017) comparable to our obtained result. These above-mentioned reports suggested that the *AP1* genes maintain the floral meristem identity function in Poaceae.

AtAP1 and *AtFUL* were found upregulated in *p35S::BtMADS14* Arabidopsis plants compared to WT (**Figure 27B**). Similar report was presented when *PpMADS2* and *PeMADS2* was overexpressed in Arabidopsis (Lin et al., 2009; Zhang et al., 2021b). This upregulation of *AtFUL* and *AtAP1* might be due to the binding of PpMADS2 protein in the CArG box of these genes. Similar DNA-protein interaction could be observed in transgenic Arabidopsis overexpressing wheat *TaVRN1* (Adam et al., 2007). Another interesting fact is that the rosette leaf numbers were significantly increased in the *p35S::BtMADS14* transgenic plant (**Figure 25A**). This result was supported by the overexpression of *Like-AP1* (*LAP1*) in hybrid aspen plant (*Populus tremula* × *Populus tremuloides*, Azeez et al., 2014).

The ectopic expression of *BtFT1* and *BtFT3* reduces the vegetative cycle approximately 7 days in transgenic Arabidopsis plants without hampering the normal floral morphology (**Figure 21**). Similar phenotypes were observed when rice *Hd3a* and *RFT1* were overexpressed in rice itself

(Kojima et al., 2002; Pasriga et al., 2019). Early flowering was also promoted in transgenic Arabidopsis overexpressing *Hd3a* (Kojima et al., 2002). Similarly, early flowering occurs when *BdFT1* of *Brachypodium* and *TaFT-B1* of *T. aestivum* were ectopically expressed in *Brachypodium* (Lv et al., 2014). In order to identify the potential target genes of *BtFT1* and *BtFT3*, expression of the downstream gene *AtAP1* was measured, which was found upregulated in both the overexpressed plants compared to WT (**Figure 23C**). Similar upregulation of *AP1* was observed in transgenic Arabidopsis plants when *FT* was overexpressed (Andrés et al., 2015).

6. Conclusion and future directions

Bamboo is a perennial plant demonstrating extremely elongated vegetative phase (up to 150 years) until the inception of flowering takes place. Here flowering and seed setting is followed by death of the plant, which is known as monocarpy. Limited studies have been conducted to explore the genetic architecture that controls such unique flowering behaviour. Being the first bamboo genome to be sequenced, *Phyllostachys edulis* remained to be a popular choice to study many important aspects of bamboo biology including flowering. However, majority of the commercially important bamboos found in India are tropical and *Bambusa tulda* emerged as a model system for tropical bamboos. Previous studies have provided information on genes related to photoperiodic pathway and their expression pattern in different tissues and light regimes. However, transgene mediated confirmation of their function remained elusive. Also, status of other established flowering pathways such as vernalization and their functional role need to be investigated in bamboos. The main aim of this study is to fill some of these gaps that still exists in this area of research. Therefore, this study focussed on the identification, sequence characterization of vernalization pathway (*VIL1, VIL2, VIL3, VIL4* and *Ghd7*) and *MADS-box* gene families, specifically *AP1* (*MADS14*, *MADS15*, *MADS18*, *MADS20*) in sequenced bamboos and their expression characterization in *B. tulda* in selected tissues, floral organs, seasons and diurnal conditions. The study also focussed on the heterologous validation of *BtMADS14*, *BtFT1* and *BtFT3* in *Arabidopsis thaliana* to decipher their flower specific roles. In a few selected cases, expression of downstream genes was analysed to identify their possible transcriptional targets.

Future studies may involve analyses on promoter regions and their detailed functional characterization to study role of promoter sequence divergence in bamboo flowering. Also, Y2H and BiFC mediated protein-protein interaction analyses will be conducted to obtain more in-depth

knowledge about all the molecular players involved here. It will be also useful to validate functions of some of these genes in another plant system such as *Brachypodium* that is phylogenetically closer to bamboo.

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8. Appendix I

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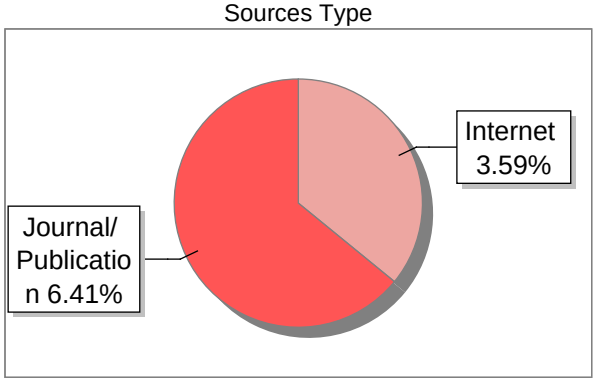

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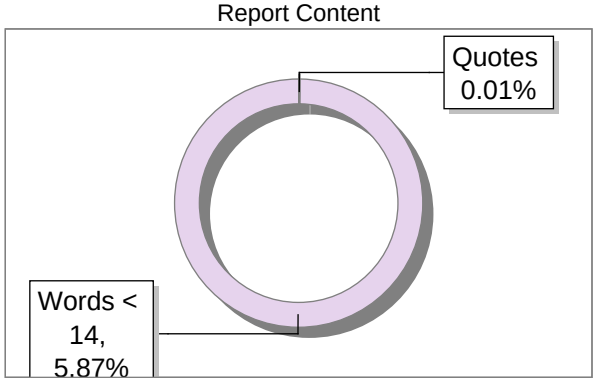

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This is to certify that ***Mridushree Basak*** *has made an oral presentation on* ***"Effect of temperature in regulation of flowering in bamboo (Bambusa tulda)"*** *in APBNS-2022, at NASC Complex, New Delhi.*

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REVIEW

Genomic insights into growth and development of bamboos: what have we learnt and what more to discover?

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Abstract

Key message **In this review, genes and transcripts that play important roles in the vegetative and reproductive development of bamboos along with their stress responses have been discussed and a few yet unresolved research questions have been identified.**

Abstract Bamboos, a member of the family Poaceae, sub-family Bambusoideae hosts approximately 1670 species within 125 genera and are distributed in Asia, America and Africa. Bamboos are used as food, fodder, medicine and also for construction, furniture and handicrafts. Bamboos also have potential as bioenergy plants for their fast shoot growth, attaining a final height of 5–20 m within only 2–4 months. They are less prone to diseases, can grow under a variety of environmental conditions, and can withstand a wide range of abiotic stresses. For fundamental research, the plant group can serve as a model to address interesting evolutionary and developmental biological questions related to its unusually extended flowering time and its expansion of gene families as a result of polyploidization. However, genomic, transcriptomic and proteomic research progress to date does not reflect the utility and research opportunities of this species group, although the first bamboo genome sequences of *Phyllostachys heterocycla* (=*Phyllostachys edulis*/moso bamboo) are available. The objective of this article is to review the current state of bamboo genomic, transcriptomic and proteomic research, and to highlight important research directions that can be pursued using these technologies.

Keywords Bamboo · Rhizome · Culm · Flower · Transcriptome · Genetic modifications

Introduction

Bamboos are distributed all over the globe except in Europe starting from 51° N in Japan to 47° S in South Argentina. It can grow up to an altitude of 4000 m above the mean sea level (Behari 2006). Approximately 22.0 × 106 ha of the forest areas are occupied by bamboo vegetation across the world (Zhou et al. 2011). About 1670 species of bamboos can broadly be classified into three major taxonomic groups (Das et al. 2008; Kelchner 2013). However, the hotspots of species richness are mainly located in the Asia–Pacific region and South America. Molecular taxonomic analyses suggest that the sub-family Bambusoideae can be divided into three tribes, i.e., Bambuseae (palaeotropical and neotropical woody bamboos), Arundinarieae (temperate woody bamboos) and Olyreae (herbaceous bamboos, Bamboo Phylogeny Group 2012; Kellogg 2015; Soreng et al. 2015, 2017). Among them Bambuseae is the largest group containing 784 tropical woody genera under 7 subtribes. The palaeotropical bamboos are usually found in tropical regions of Africa, Madagascar, Sri Lanka, India, Southern Japan, Southern China and Oceania (Fig. 1), while the neotropical members are distributed in Southern Mexico, Argentina, Chile and West Indies.

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RESEARCH ARTICLE

Identification, expression analyses of *APETALA1* gene homologs in *Bambusa tulda* and heterologous validation of *BtMADS14* in *Arabidopsis thaliana*

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Abstract
Bamboos belong to the grass family Poaceae, sub-family Bambusoideae and possess many interesting developmental features including a long vegetative period before flowering. Previously, transcriptome based analyses have identified differentially expressed transcripts in flowering and vegetative tissues to predict gene clusters of functional relevance. In contrast, limited studies were conducted to characterize individual genes to decipher their precise role to induce flowering. This was primarily due to the unavailability of sufficient genomic resources, which has lately been overcome by the release of additional bamboo genomes. In this study, the *APETALA1* gene homologs (*MADS14*, *MADS15*, *MADS18* and *MADS20*) have been identified from five sequenced bamboo species (*Bonia amplexicaulis*, *Guadua angustifolia*, *Raddia guianensis*, *Olyra latifolia*, *Phyllostachys edulis*). In addition, *APETALA1* homologs from a tropical bamboo (*Bambusa tulda*) have been PCR amplified, sequenced and included in the analyses to widen spectrum of sampling. Assessment of their phylogenetic and syntenic relationship with related Poaceae neighbours revealed closer relationship between *MADS14* and *MADS15* members than *MADS18* and *MADS20*. Transcriptional expression patterns of *B. tulda BtMADS14*, *BtMADS15*, *BtMADS18* and *BtMADS20* in vegetative and floral tissues indicated a possible role of *BtMADS14* and *BtMADS15* in flower induction and differentiation, while *BtMADS18* might be associated with seed development. Total 24 proteins were predicted to interact with the *Phyllostachys edulis* homolog of BtMADS14 protein and 8 of them were members of the MADS-box family. The *p35S::BtMADS14* overexpressing Arabidopsis plants flowered 8–10 days earlier than the wild type plants suggesting its possible involvement in the floral induction of *B. tulda*.

Keywords Poaceae · Flowering · *MADS*-box · Gene expression · Overexpression

Abbreviations

AP1	Apetala1
BOP clade	Bambusoideae Oryzoideae Pooideae clade
MIKC	MADS domain, Intervening domain, Keratin-like domain, C-terminal domain
RT-qPCR	Quantitative Reverse Transcription Polymerase Chain Reaction

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Introduction

Flowering is a central transition process in the life cycle of an angiosperm. The switch from the vegetative to reproductive phase is initiated by the conversion of the vegetative apical meristem to an inflorescence meristem. This process is controlled by multiple pathways influenced by different environmental cues such as temperature, day length, precipitation pattern and also endogenous biochemical factors such as sugar and hormone (Yu et al. 2020). Extensive studies performed on the model dicot (e.g., *Arabidopsis thaliana, Antirrhinum majus*) and monocot plants (e.g., *Oryza sativa*, *Brachypodium distachyon*) revealed that network of flowering genes are complex, yet varying in different plants.

Floral organ differentiation is a post floral induction process that regulates proper development of the four floral whorls and is controlled by a set of genes, whose functioning can be elucidated by the well-known ABCDE model.

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